KUHNEN & WACKER
Patent- und Rechtsanwaltsbüro

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Official File No. : 93 909 679.8 / 0 656 786
Our Reference : 57/AL21K01/EP
Owner/Applicant: Novogen Research Pty Ltd.
Subject : Opposition against EP 0 656 786

On behalf of

ALSITAN GmbH & Co. KG
Am Bühl 16 – 18
86926 Greifenberg
Germany

we herewith lodge

OPPOSITION

against European Patent No. 93 909 679.8 / 0 656 786 in the name of

Novogen Research Pty Ltd.
140 Wicks Road
Nort Ryde, NSW 2113
Australia

Zur Kasse €610,-(A)

and having the title:

USE OF ISOFLAVONE PHYTO-OESTROGEN EXTRACTS OF SOY OR CLOVER

It is requested to:

1. revoke the patent as a whole, particularly since the subject matter of this European Patent does not appear to be patentable in accordance with Articles 52 through 57 EPC;

2. to fix a date for Oral Proceedings if the EPO should not be in a position to decide according to the request under 1 in written proceedings.

The opposition fee (EUR 610,00, Code No. 010) is to be deducted from our account No. 2800.0913 (Association No. 150) according to the attached voucher.

The opposition is based on the grounds of Art. 10(a) and 100(b) EPC.

Further to the prior art cited on the patent as published, the following prior art documents support the opposition and are herewith submitted:

D1 Scand J Clin Lab Invest 1990; 50, Suppl 201: 3-23, „Western diet and Western diseases: some hormonal and biochemical mechanisms and associations"

D2 Japanese Unexamined Patent Application (Kokai) No. 62(1987)-126186 and its English translation which is referred to in the following

D3 The Lancet, Vol. 339, May 16, 1992, 1233, „Dietary phyto-oestrogens and the menopause in Japan"

D4 Japanese Unexamined Patent Application (Kokai) No. 61(1986)-246124 and its English translation which is referred to in the following

D5 BMJ, Vol. 301, October 20, 1990, 905-906, „Oestrogenic effects of plant foods in postmenopausal women"

D6 Reproductive Toxicology Review, 1989, Vol. 3, No. 2, 81-89

D7 Food additives and contaminats, 1985, Vol. 2, No. 2, 73-106

SUBSTANTIATION:

Disclosure of the specification

The specification of the opposed patent to a large extent only reiterates facts about isoflavones from the prior art without any citations of relevant references. This relates to the occurrence in different plants, specific compounds and the ratio in which these specific compounds occur and usually are obtained from these plants, and the alleged effects of a diet rich in isoflavones as already published in the prior art.

I. Insufficient disclosure (Art. 100(b) EPC in connection with Article 83 EPC)

The opposed patent very broadly claims the use of an isoflavone phyto-oestrogen extract of soy or clover (2 alternatives), for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer (3 further alternatives) in claim 1 and in dependent claims 2 to 11.

However, there is no evidence and no proof, neither in the opposed patent nor in the prior art that enables a person of ordinary skill to practice the alleged invention over the entire and very broad range as claimed. This is, however, required by the EPC and the relevant case law, respectively. Therefore, the opposed patent lacks a sufficient disclosure and, therefore, contravenes Article 83 EPC.

In detail, the specification does not go beyond what is already known and obvious. Moreover, in the examples it is only shown that there is a certain effect of a diet containing soy hypocotyl – which is not a unit dosage form – on cholesterol level in humans. One woman out of a group of eight and a total of 15 test persons allegedly reported a „substantial amelioration of her begnine breast disease problem associated with mid-cycle swelling and tenderness" and another woman reported „regularisation of her menstrual cycle and reduced menstrual bleeding" (EP 0 656 786, para [0085]). These „effects" may have occurred for any reason and there is no evidence, like for instance proven in a double blinded study, that proves these „effects" to be the result of a treatment with isoflavones.

The term „treatment" comprises prophylactic, curative, and palliative treatment and is therefore generally wide in scope. The opposed patent does not disclose a

teaching nor any proof for a teaching according to which the indications as claimed can effectively be treated in the broad sense as claimed, at all.

It is in particular not disclosed how any of the indications are to be treated and the only teaching disclosed is to administer a dosage of an isoflavone cocktail contained in plants in the form of parts of the plant, soy hypocotyls, in an amount within the range of an ordinary, albeit isoflavone rich diet. No teaching relating to specific conditions, e.g. relating to the remission of prostate cancer, alleviation of the symptoms of pre-menstrual syndrome, symptoms associated with menopause or other conditions apart from the reduction in cholesterol levels in blood is disclosed in the opposed patent.

In fact, to enable one of ordinary skill to be in the position to practice the alleged invention over the broad range claimed. This requires more than the instant disclosure. There is for instance no teaching disclosed that relates to the dosage or administration regimen for the treatment of specific indications that fall within the claimed range of indications and the examples, as far as they relate to the effect of an administration of isoflavones, do not cover the indications as claimed. Even more, the entire specification is absolutely silent regarding any definition of the term „symptoms associated with menopause" or with respect to what exactly is to be understood by "pre-menstrual syndrome" so that not even the exact meaning of these terms is sufficiently disclosed.

In view of the above the opposed patent already has to be revoked because of lack of sufficient disclosure.

II. Lack of inventive step (Art. 100(a) EPC in connection with Article 56 EPC)

Claim 1

1. Document D1 in combination with Document D2

Document D1, a review article with the title „Western diet and Western diseases: some hormonal and biochemical mechanisms and associations", discloses on pages 13 and 14 that there is a suggested effect of dietary isoflavones to slow down the speed of cancer cell growth. In this connection prostate cancer is explicitly mentioned and also the low mortality in Japan and some other Asian countries, despite the same incidence of latent small or non-infiltrative carcinomas. This is particularly attributed to the presence

of „high levels of isoflavonic phyto-oestrogens in the traditional diet of Japanese men".

Thus, document D1 already discloses the beneficial effects of isoflavones in relation to the indication prostate cancer. Therefore, D1 is considered the closest prior art. This particularly applies since this document very clearly discloses isoflavone phyto-oestrogens deriving from soy products as being responsible for this beneficial effect on the indication of prostate cancer.

Starting off from document D1 the problem underlying the opposed patent is to provide for an easy access to isoflavones in order to make the same readily available and to facilitate their uptake for obtaining the beneficial effects associated therewith more easily.

In order to solve the above problem the person of ordinary skill in the art would turn to document D2 because D2 discloses a process for producing isoflavone derivatives that have known pharmacological effects (page 1) and that will be provided as pharmaceuticals (page 2) or, in other words, as medicaments.

The combination of documents D1 and D2 therefore obvious to those of ordinary skill and leads to the use an isoflavone phyto-oestrogen extract of soy (D2, p. 1, „Scope of Claims for Patent") or clover, for the manufacture of a medicament (D2, p. 2, 1st para, „pharmaceutical") for administration in unit dosage form or, in other words, the way medicaments are commonly administered, for the treatment of pre-menstrual symptom, symptoms associated with menopause, or prostate cancer (D1, pages 13 – 14).

In view of the combination of documents D1 and D2 claim 1 of the opposed patent lacks the required inventive step.

According to a different approach document D2 could be taken as the closest prior art because it discloses the majority of the features of claim 1. In this case the problem for those of ordinary skill is to find indications for treatment with a medicament in unit dosage form made from an isoflavone phyto-oestrogen extract.

Since D1 discloses the indication prostate cancer the combination of features of claim 1 of the opposed patent is also obvious when starting off from D2 as closest prior art.

2. Document D3 in combination with document D2

Document D3 discloses that high levels of isoflavonoid phyto-oestrogens associated with the intake of soy products may explain the relative absence of „hot flushes and other menopausal symptoms" in Japanese women (D3, last para of article).

Starting from D3 as closest prior art because this document does mention the indication (symptoms associated with menopause or simply menopausal symptoms) and also the treatment (the intake of high levels of isoflavonoid phyto-oestrogens) the problem to be solved appears to be to provide an easy to administer form of isoflavonoid phyto-oestrogens.

Again, the combination with the disclosure of document D2 leads to the subject-matter of claim 1 of the opposed patent in an obvious way and for the same reasons set forth for the combination of documents D1 and D2, albeit for a different indication.

Therefore, claim1 also lacks the required inventive step in view of the combination of documents D3 and D2.

Also, and for the similar reasons as for the previous combination of documents D2 with D1 those of ordinary skill in the art could start off from document D2 as closest prior art and would arrive at the subject-matter of claim 1 of the opposed patent without an inventive step because document D3 teaches the effect of high levels of isoflavonoid phyto-oestrogens on menopausal symptoms in postmenopausal women.

Consequently, claim 1 of the opposed patent also lacks an inventive step over the combination of D2 with D3.

3. Combination of document D4 with document D2

Document D4 discloses the use of an isoflavone, specifically genistein, as a carcinostatic agent and that this compound can be isolated from clover (D4, page 1). Further, this document discloses the administration of genistein as active component at a dose of 200 to 1,000 mg per adult per day (D4, page 8, lines 1 and 2), in different unit dosage forms and in preparations or

dosage forms as compositions comprising dietary suitable excipients (D4, page 8, last para).

When considering document D4 as closest prior art because it discloses the general indication for the treatment of carcinomas and, apart from the disclosure of a formulation as a medicament in unit dosage form, also indicate a source for obtaining the compound, clover, those of ordinary skill would consider the disclosure of document D2 in order to solve the problem of identifying and providing a convenient and economically feasible source of the carcinostatic agent and thereby arrive at the subject-matter of claim 1 of the opposed patent because document D2 discloses an isoflavone phyto-oestrogen extract of soy for the manufacture of a medicament.

The specific indication, prostate cancer, does not require to be mentioned because it is well within the scope of routine experimentation to screen carcinoma cell lines for a specific indication. In particular, since D4 already discloses a number of different assays for different carcinoma cell lines.

As already indicated, although such kind of experimentation may require some effort with respect to the number of assays that have to be run, the required laboratory space and equipment, it is every day work in pharmaceutical research and does not exceed ordinary skill.

Therefore, the subject-matter of claim 1 of the opposed patent is not inventive in view of the combination of documents D4 and D2.

4. Combination of document D5 with document D2

Document D5 can also be considered the closest piece of prior art because it discusses the effect of phyto-oestrogens, isoflavones, coumestans, and lignans (D5, page 905, left col.) on postmenopausal women (D5, Subjects, methods, and results) from soya flour and red clover sprouts (D5, page 906, left col.) and gives a strong indication towards further investigation or research in this area (D5, page 906, end of article).

Setting off from this document those of ordinary skill in the art would recognise the beneficial effect of the compounds disclosed in D5 on symptoms associated with menopause and arrive at the combination with the disclosure of document D2 for the same reasons and for solving similar or

the same problems as mentioned hereinbefore without employing an inventive step.

Thus, the subject-matter of claim 1 of the opposed patent is also lacking an inventive step in view of the combination of D5 with D2.

5. Combination of document D6 with document D2

Document D6, a review on „Reproductive and general metabolic effects of phyto-oestrogens in mammals" discloses the application or rather administration of phyto-oestrogens in traditional Chinese herbal medicine for the treatment associated with menopause (D6, page 88, right col., one but last para) and, therefore forms the indication for the combination with the disclosure of document D2 in order to solve the problems already mentioned hereinbefore.

In view of this obvious combination of documents D6 and D2 claim 1 of the opposed patent lacks an inventive step.

6. Combination of document D7 with document D2

In a similar way as above the combination of documents D7 and D2 leads to the subject-matter of claim 1 of the opposed patent. Since D7 is a review article on naturally occuring oestrogens in food, covering in particular isoflavones and isoflavone glucosides (D7, page 75 and following) and coumestans (D7, page 85 and following), the subject-matter of claim 1 of the opposed patent is without any inventive merit because document D2 provides sufficient information for those of ordinary skill in order to achieve the obvious – the use of an isoflavone phyto-oestrogen extract of soy or clover, for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer.

Claims 2 to 11

Claims 2 to 11, directly or indirectly, depend on claim 1 of the opposed patent. None of these claims contains a feature that, when included in claim 1, would render the claim inventive.

However, the features of these claims are either known from the prior art or arbitrary.

In particular, claim 2 recites that the medicament further comprises at least one dietary excipient. This feature is in accordance with general practice in the field of formulating pharmaceutically active substances. In order to prevent local overdosing and for numerous other reasons, like continuous development of blood levels, easier manufacturing and the like active substances are only exceptionally formulated as pure compounds.This feature is also disclosed in document D4 (D4, page 9, last para).

Claim 3 requires that the isoflavone phyto-oestrogen is extracted from soya. This only specifies one of the possible alternatives of claim 1 of the opposed patent and is void of any inventive contribution to the already claimed subject-matter.

Soya hypocotyls as basis for the isoflavone phyto-oestrogen extract as claimed in claim 4 is derivable from document D7, where the relative high abundance of isoflavones in the hypocotyl is disclosed (D7, page 83, 3rd sentence of last para). It is merely obvious to isolate the desired compounds from that part of the plant which contains most of them in order to work efficiently and economically.

Clover as source of the isoflavone phyto-oestrogen extract according to claim 5 is mentioned in a number of the documents cited hereinbefore and is a generally known source of isoflavones, for instance in D4, page 1, "prior art", and D7, page 74, table 1.

Claim 6 only reiterates the form in which isoflavones occur in plants, as a mixture of related compounds, as glycosides thereof, or metabolites or derivatives thereof and therefore necessarily constitute the isoflavone phyto-oestrogen extract.

The ratio of compounds as claimed according to claim 7 is merely arbitrary and no teaching relating to an inventive contribution can be derived from the entire patent.

The dosage claimed according to claim 8 is within the range of a common diet comprising isoflavones and therefore rightout obvious.

Dosage regimens and period of administration are easily determined by routine experimentation by those of ordinary skill an can render the subject-matter of non of the claims inventive.

Since coumestans, lignans and flavones, including isoflavones, do occur together in nature their presence in the extract of claim 1 is natural and a consequence of preparing a simple isoflavone phyto-oestrogen extract and therefore not inventive.

The specification of a unit dosage form without any specific benefits can by no means render the claimed subject-matter inventive.

For all of the above the initial request to fully revoke the opposed patent is justified. Consequently, the opposed patent will have to be revoked in its entirety.

Therefore, the requests mentioned above are fully justified, and the patent in dispute has to be revoked as a whole.

Patent attorney
M. Andrew May
(Association No. 150, ☎ 08161 608 314)

Encs.

D1 Scand J Clin Lab Invest 1990; 50, Suppl 201: 3-23, „Western diet and Western diseases: some hormonal and biochemical mechanisms and associations" (2-fold)

D2 Japanese Unexamined Patent Application (Kokai) No. 62(1987)-126186 and its English translation which is referred to in the following (2-fold)

D3 The Lancet, Vol. 339, May 16, 1992, 1233, „Dietary phyto-oestrogens and the menopause in Japan" (2-fold)

D4 Japanese Unexamined Patent Application (Kokai) No. 61(1986)-246124 and its English translation which is referred to in the following (2-fold)

D5 BMJ, Vol. 301, October 20, 1990, 905-906, „Oestrogenic effects of plant foods in postmenopausal women" (2-fold)

D6 Reproductive Toxicology Review, 1989, Vol. 3, No. 2, 81-89 (2-fold)

D7 Food additives and contaminats, 1985, Vol. 2, No. 2, 73-106 (2-fold)

Voucher

Prostate cancer !

D1

Scand J Clin Lab Invest 1990; 50, Suppl 201: 3–23

Western diet and Western diseases: some hormonal and biochemical mechanisms and associations

HERMAN ADLERCREUTZ

Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, Helsinki, Finland

Adlercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest 1990; 50 Suppl 201: 3–23.

Breast cancer, prostate cancer, coronary heart disease and colon cancer belong to the so-called Western diseases and a general opinion is that diet is a significant or even the main factor increasing incidence and mortality of these diseases in the Western world. This review describes studies carried out in this department for about 10 years, many in collaboration with scientists abroad, and with the aim to clarify some of the connections between the diet and sex hormone, lipid and bile acid metabolism. A Western-type diet elevates plasma levels of sex hormones and decreases the sex hormone binding globulin concentration, increasing the bioavailability of these steroids. The same diet results in low formation of mammalian lignans and isoflavonic phytoestrogens. These diphenolic compounds seem to affect hormone metabolism and production and cancer cell growth by many different mechanisms making them candidates for a role as cancer protective substances. The precursors of these diphenols are to be found in fiber-rich unrefined grain products, various seeds, beans and probably also in pulses, peas and berries. Some types of fiber seem to influence sex hormone and bile acid metabolism mainly by partial interruption of the enterohepatic circulation, by alteration of intestinal metabolism and by increasing fecal excretion of these compounds. The sex hormone pattern found in connection with a Western-type diet is prevailing in the breast cancer patients, but is only partly a result of the diet.

Key words: breast cancer, prostate cancer, colon cancer, coronary heart disease, diet, fiber, lignans, isoflavones, estrogens, androgens, sex hormone binding globulin, dihydrotestosterone, bile acids, feces

Herman Adlercreutz, M.D., Professor, Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, SF-00290 Helsinki, Finland

Breast cancer (BC), prostate cancer (PC) and endometrial cancer (EC) belong to the group of hormone-dependent cancers which in addition to colon cancer (CC), coronary heart disease (CHD) and some other diseases are called Western diseases because their incidence and mortality are high in the Western world compared to countries in Asia and South and East Europe [1–3]. In migrant studies an increased risk for Western diseases has been found to be related to a change towards a Westernized diet [4–9]. Migrants from Asia, Africa or East Europe to U.S.A. or Australia have

originally consumed a low-fat vegetarian or semi-vegetarian diet containing large amounts of unrefined carbohydrates. Most of these migrants and their children rapidly adopt a diet rich in calories, fat and proteins and low in complex carbohydrates and fiber [10] and their hormone [11, 12] and lipid levels change towards a Western pattern, increasing the risk for hormone-dependent cancer and CHD. Interestingly, migrants from a high risk colon cancer area (Scotland) to Australia experience a reduced risk for colon cancer [9].

Furthermore Hill et al. [13] postulated that a Western-type diet increases the concentration and metabolism of fecal bile acids (FBA) and neutral sterols (FNS), increasing the risk for CC. In the majority of the population studies carried out this hypothesis has rendered support with regard to the concentration but not with regard to the metabolism of FBA and FNS [review in 14]. On the other hand many animal experiments and *in vitro* tests have shown that free bile acids are cocarcinogenic or comutagenic [15, 16] but that the aminoconjugated bile acids may be inactive [16] in this regard [reviews in 14, 17]. It is believed that secondary bile acids are more toxic than primary ones and that a high litocholic acid (LCA) to deoxycholic acid (DCA) ratio is a CC risk factor [18–20].

Because of the obvious relationship between Western diet and Western diseases it has been postulated that this type of diet by some biochemical or other mechanisms may alter hormone production, metabolism or action at the cellular level increasing the risk for hormone-dependent cancer. Furthermore it has been suggested that the dietary composition may influence transit time of the intestinal content, fecal bulk and intestinal microflora and its environment causing alterations in concentration and metabolism of hormonal steroids, bile acids, neutral sterols, carcinogens and procarcinogens increasing the risk of CC and BC. Particularly in women, who have a much higher incidence of hormone-dependent cancer than men, diet has been suggested to be the main single determinant in the etiology of these cancers.

It is, however, very difficult to separate the effects of various single macro- or micronutritients on any biochemical event or steroid hormone or bile acid pattern or level. This is not only due to difficulties in the accurate recording of the diet, but also to the great variability in dietary intake during different seasons and even different parts of the week and the variability of hormone and steroid levels, particularly in women. Special efforts have to be made to standardize the conditions for sampling and to use reliable hormone assay methods and the recording of the diet must be carried out during sufficiently long time [12].

The following review will summarize and discuss results of our studies on the connection between diet and Western diseases. Many of these investigations are the result of collaborations with scientists abroad and some results discussed have not yet been published. The review will deal with some newly discovered mechanisms of dietary effects on sex hormone and intestinal bile acid metabo-

TABLE I. Abbreviations and trivial names of steroids and other abbreviations used in the text.

A	Androstenedione
BC	Breast cancer
CC	Colon cancer
CHD	Coronary heart disease
DHEAS	Dehydroepiandrosterone sulfate
Da	Daidzein
DCA	Deoxycholic acid
5α-DHT	5α-Dihydrotestosterone
EC	Endometrial cancer
End	Enterodiol
Enl	Enterolactone
E2	Estradiol
E3	Estriol
E1	Estrone
E1S	Estrone sulfate
Eq	Equol
FBA	Fecal bile acids
FNS	Fecal neutral sterols
For	Formononetin
FE2	Free estradiol
FT	Free testosterone
Gen	Genistein
2-OHE1	2-Hydroxyestrone
4-OHE1	4-Hydroxyestrone
%FT	Percentage free testosterone
%FE2	Percentage free estradiol
PC	Prostate cancer
LCA	Litocholic acid
LH	Luteinizing hormone
Mat	Matairesinol
SHBG	Sex hormone binding globulin
T	Testosterone

lism and in addition with some interesting associations between the various diseases. Further support for the previously proposed extension [21] of the "fiber hypothesis" of Burkitt & Trowell [see 10] has now been obtained and will be discussed including not only BC and CC but also other Western diseases.

EFFECT OF VARIOUS MACRONUTRITIENTS ON SEX HORMONE METABOLISM

Effect of fiber

The development of a radioimmunological chromatographic method for the assay of the very low amounts of estrogens present in feces of men and nonpregnant women [22] made it possible for the first time to obtain a complete view of the effect of diet on the enterohepatic circulation of estrogens in man.

A high intake of fiber in premenopausal women increases fecal wet and dry weight, which correlates positively with all three unconjugated estrogens and total estrogens in feces [23]. In the same study also postmenopausal women were investigated (H. Adlercreutz, E. Hämäläinen, S.L. Gorbach, B.R. Goldin, J.T. Dwyer, M.N. Woods, unpublished results) and the same results were found. Furthermore, in the postmenopausal women we found positive associations between total fiber and grain fiber intake, and fecal estrone (E1) and estradiol (E2) excretion (list of abbreviations in Table I). Fat intake on the other hand seems to have a negative association with fecal excretion of estrogens [24] and therefore the dietary fat/fiber ratio of the postmenopausal women living in Boston shows highly significant negative correlation with fecal estrogen excretion (above-mentioned unpublished study). It is suggested that the dietary fat/fiber ratio determines the degree of interruption of the enterohepatric circulation of steroids, but the type of fiber plays also a significant role (see below).

In premenopausal women fecal weight and fecal estrogen excretion was found to correlate negatively with urinary estrogen excretion [23]. Particularly important was the observation of a negative correlation between fecal estriol (E3) and urinary E3-3-glucuronide (E3-3G) excretion. Urinary E3-3G is a specific metabolite of the intestinal mucosal cell and the end-product of estrogen metabolism and therefore a good indicator of the extent of the enterohepatic circulation of estrogens, particularly of E3 and other 16-hydroxylated and polar estrogens in man [25]. In a study carried out in Helsinki in premenopausal women it was found that total fiber intake and grain fiber intake/kg body weight were negatively associated with the excretion of 10 of the 13 estrogens measured in urine [26].

Fecal estrogen excretion shows a negative association with plasma E1 and E2 [23] and later on a direct negative correlation between total fiber intake and plasma E1 and E2 [24] and estrone sulfate (E1S) [27] could be observed in young women. Similar findings in men have been reported, but in addition to the negative correlation between crude fiber intake and plasma E2, higher fiber intake is associated with lower plasma testosterone (T) levels [28–30]. The reason for reduced intestinal reabsorption and increased elimination of estrogens by the fecal route in subjects consuming much fiber seems to be the larger fecal bulk and decreased concentration of intestinal β-glucuronidase [21, 23, 25]. The latter phenomenon reduces hydrolysis of the biliary steroid conjugates, an event necessary for their reabsorption. Some fibers have also the property of binding sex hormones, particularly non-polar estrogens [31, 32].

Preliminary results in the large study in Helsinki, called the "Finlandia study" revealed significant positive correlations between intake of total fiber, vegetable fiber and fiber from fruits and berries and plasma sex hormone binding globulin (SHBG) and negative associations between the intake of the same fibers and plasma % free estradiol (%FE2). Furthermore, total fiber, grain fiber and vegetable fiber intake correlated negatively with plasma % free testosterone (%FT) [33, 34]. The new results obtained in in postmenopausal Boston women [23, 24] agree well with the above-cited publications in that significant negative correlations were found between intake of total fiber, grain fiber and non-grain fiber and plasma androstenedione (A), T, FT [35] and E1. In addition intake of fruit and vegetable fiber and grain calories correlated negative-

ly with plasma E1 [estrogen results unpublished, see 27].

It may be concluded that high fiber intake is associated with low levels of sex hormones in plasma, high SHBG and low %FE2 and %FT causing a reduction in the bioavailability of the hormones, which theoretically would reduce the risk of hormone-dependent cancer. The proposed mechanisms involved in changing the SHBG level will be discussed in the sections on dietary protein, and lignans and isoflavonic phytoestrogens.

Effect of protein

Most of the studies on the effect of protein intake on hormone metabolism have been carried out by altering the protein/carbohydrate ratio of the diet. Using this technique it was found that a high dietary protein/carbohydrate ratio decreases the plasma level of SHBG and T and that a low ratio has the opposite effect [36, 37]. Furthermore a high protein diet considerably diminished 4-ene-5α-reduction of T and enhanced 2-hydroxylation of E2 [38, 39]. By measuring the estrogen profile in urine by capillary GC-MS in premenopausal women [40, 41] we could recently confirm that a high dietary protein/carbohydrate ratio results in high urinary excretion of catecholestrogens. A new finding was that the dietary protein/carbohydrate ratio is highly significantly and positively associated with the urinary 2-OH-E1/4-OH-E1 ratio. Furthermore the lowest mean ratio (= 3.6) was found in vegetarians, followed by the omnivores (= 4.3) and the highest was found in the BC patients (= 7.1) (BC *vs.* vegetarians $p < 0.005$; BC *vs.* omnivores $p < 0.02$), who had the highest dietary protein/carbohydrate ratio due to low grain intake. It may be mentioned that this ratio was recently found to be 2.0 in the same Oriental migrant women in Hawaii [42], which were previously studied by us [24].

Effect of carbohydrates

In the above section the effect of changes in the dietary protein/carbohydrate ratio was discussed. Some further information as to the possible effect of carbohydrates on sex hormone metabolism derives from studies in which dietary intake of various macro- and micronutritients were correlated with plasma and urinary hormone levels.

Recently we found that postmenopausal women living in Boston showed significant negative associations between carbohydrate intake and plasma T, E1 and E2 [35,43]. Furthermore in the same study the intake of grain calories showed negative correlations with plasma A, T, DHEAS, and E1. The intake of carbohydrates also showed a weak but significant positive correlation with fecal E1 excretion (estrogen results unpublished).

In the corresponding Finnish study in 33 premenopausal women [40–42], studied twice during a year, we found some other interesting correlations between carbohydrates and sex hormones. Urinary 2-OH-E1/4-OH-E1 ratio correlated positively with protein/carbohydrate ratio of the diet and negatively with carbohydrate, starch, total fiber and grain fiber intake. Urinary 4-hydroxyestrone excretion correlated positively with total and grain fiber intake and plasma SHBG and negatively with %FE2 and %FT. Starch intake was negatively associated with urinary E3-3-glucuronide, the specific marker of the enterohepatic circulation of estrogens, suggesting partial interruption of this circulation in subjects with high starch intake. Carbohydrate intake was negatively associated with plasma E1S, the mean level of which was highest in the BC group. Plasma DHEAS on the other hand was strongly positively associated with plasma E1S, and less strongly with %FE2 and negatively associated with urinary 16-hydroxylated estrogens and enterolactone (Enl) [27]. Enl mainly derives from precursors in grain and its urinary excretion reflects both the intake of fiber in general [44] and whole-grain products in particular. The results indicate that it is difficult to separate the effect on hormone metabolism of complex carbohydrates from that of fiber.

Effect of fat

Oriental women living in East Asia and at low risk for BC consume a very low-fat diet (usually < 20 % of calories). Studies on the urinary excretion of E1, E2 and E3 have shown that they excrete lower amounts of E1 and E2 and similar amounts of E3

compared to women in Western countries [24, 45, 46]. In other studies in vegetarians living in Western societies the picture has not been so clear, but there has been a trend towards lower urinary E1 and E2 values and similar or slightly higher E3 values in the vegetarians [47, 48]. Thus a vegetarian or semivegetarian diet seems to be associated with relatively high E3 formation. The simultaneously higher fecal excretion of E3, however, reduces urinary E3 levels leading to varying quantitative results for E3 in urine, depending mainly on the nature of the fiber in the food and the quantity of both dietary fiber and fat. Simultaneously there seems to be a reduction in the relative concentration of 2-hydroxyestrogens, particularly in Oriental women and a relative increase in 4-hydroxylation [41, 42], which means that the main metabolic pathways in these women unexpectedly seems to lead to biologically more active estrogens. However, it must be remembered that their plasma and urinary E1 and E2 levels were shown to be low [24] and the net biological estrogen effect may in any case be less. It has also been shown that the luteal phase E2 values are lower in young women following a low-fat diet for 2 months [49].

Women living in Africa consuming low-fat habitual diets [50] and Oriental migrants in Hawaii [24] have low plasma androgen levels compared with women on a Western diet. These observations are in agreement with the results obtained in postmenopausal omnivorous and vegetarian women and postmenopausal women with BC showing the lowest plasma A, T, %FT, %FE2 and DHEAS and highest SHBG (after correction for weight) in the vegetarian women, who had the lowest dietary fat/fiber ratio of the three groups [35, 43] The lower DHEAS in vegetarians is in agreement with recent results showing that plasma E1S levels are lower in women on a low-fat high-fiber diet compared to a typical Western diet [51] because the levels of these sulfates show a significant association [27 and unpublished results]. In correlation analysis a Western-type diet was found to be associated with the hormonal pattern observed in the postmenopausal women with BC, but this was obviously not entirely due to the diet [35].

It seems justifiable to conclude that a high protein and fat and low grain, complex carbohydrates and fiber intake leads to higher plasma levels of biologically active sex hormones and lower SHBG, with a clear tendency to lower 16α– and 16β-hydroxylation [42] and higher 2-hydroxylation of estrogens and higher urinary 2-hydroxy-E1/4-hydroxy-E1 ratio. The possible role of these alterations of hormone levels as etiological factors in hormone-dependent cancer will be discussed below. It should be mentioned that opposite results with regard to 16α-hydroxylation of estrogens and fat intake have been published [52, 53], and these results will be discussed in the section on BC.

LIGNANS, ISOFLAVONES, AND SEX HORMONE METABOLISM

Since the detection and identification of mammalian and later also of plant lignans and isoflavonic phytoestrogens in the human organism, many studies on their biological role in health and disease have been carried out. Several reviews [33, 54–56] on the topic have recently been published. These diphenolic compounds occurring in high amounts in the organism have numerous different biological activities of which most seem to make them candidates for a role as protective substances with regard to cancer and particularly hormone-dependent cancers [12, 21, 33, 34, 54, 56–64].

To date 15 lignans and isoflavonic phytoestrogens, all diphenolic in character, have been identified in human urine and some of them also in other biological materials [54, 56, 65, 66]. Of these 7 can now be measured by combined capillary gas chromatography–mass spectrometry utilizing the selective ion monitoring technique and isotope dilution mass spectrometry using deuterated internal standards [58, 67]. The lignans enterolactone (Enl), enterodiol (End) and matairesinol (Mat) and the isoflavonic phytoestrogens daidzein (Da), equol (Eq), O-desmethylangolensin (O-Dma) and genistein (Gen) have all weak estrogenic activity, but antiestrogenic activities have also been described [reviews in 54, 56]. Many plant lignans have been shown to have anticarcinogenic, antiviral, bactericidic and antifungal activities. In collaboration with Dr Larry Vickery (Irvine, California) it was shown that Enl and a theoretical

intermediate between Mat and Enl are moderate inhibitors of placental aromatase and compete with the natural substrate androstenedione for the enzyme. Enterolactone was also able to inhibit aromatase intracellulary in cell cultures suggesting that these compounds may function as natural aromatase inhibitors. Other experiments show that these diphenols are readily transferred from cell culture media into the cells and that they may inhibit cancer cell growth, because antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast cancer cell line, ZR-75-1, were reported [59]. Furthermore, inhibitory effects of such compounds on mitogen-induced proliferation of human peripheral blood lymphocytes were demonstrated [60].

Genistein, one isoflavonic compound identified by us in human urine is a specific inhibitor of tyrosine-specific protein kinases [61-64]. Protein-tyrosine kinase activity is associated with cellular receptors for epidermal growth factor (EGF), insulin, insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF) and mononuclear phagocyte growth factor (CSF-1), suggesting that the enzyme plays a role for cell proliferation and transformation. The enzyme has also been associated with oncogene products of the retroviral src gene family and is correlated with the ability of retrovirus to transform cells [literature in 61–64].

In collaborative studies with Dr Jim Clark and associates we have found that several plant and mammalian lignans and isoflavones compete with E2 for the rat uterine nuclear estrogen type-II binding site (unpublished results). These sites seem to constitute a component of the genome which regulates estrogen-stimulated uterine growth [68, 69]. It was found that some flavonoids like luteolin, quercetin and pelargonin inhibit E2 binding to this receptor and in this way uterine cell growth. They also inhibited growth of MCF-7 cells in culture, and *in vivo* E2 stimulation of immature rat uterus [70]. The structure of these compounds are very similar to those of the isoflavones and in fact all are diphenols. The most effective with regard to type II site binding of the diphenolic compounds found and measured by us in human urine seem to be the isoflavones daidzein and equol, but also some lignans like matairesinol, isolariciresinol and enterolactone show competition (competition observed at concentrations from 10 to 100 nmol/l). Later an endogenous inhibitor of the nuclear type II binding site was identified as being methyl p-hydroxyphenyllactate [71], which can be a metabolite of both exogenous flavonoids and tyrosine. Because this compound cannot be found in cancer tissue it was postulated that uncontrolled growth and proliferation of malignant cells is directly related not only to the permanent stimulation of nuclear type II binding sites by estrogens or other compounds, but also to very low to nonmeasurable levels of the competitive inhibitor methyl p-hydroxyphenyllactate [71]. In our opinion it seems that probably many of these phenolic compounds may have a synergistic action as it is unlikely, because of close structural similarities, that only one of them inhibits cell growth. The compound found by Markarevich et al. [71] was isolated from fetal bovine serum, probably a very rich source of many flavonoids and phytoestrogens and their metabolites. The concentration of the new monophenolic compound in biological fluids and tissues in human subjects has to my knowledge not been measured. The possible growth-inhibiting and antiproliferative role of individual flavonoids and their metabolites with regard to hormone dependent cancer is a new interesting area of research that needs much further studies.

Of the isoflavones the strongest estrogens are Eq and Gen, but they are still very weak estrogens compared to E2 and E1. It is unlikely that all their other biological effects are related to their estrogenicity. Quantitative results indicate that lignans and isoflavonic phytoestrogens are normal constituents of human urine and are excreted in large amounts particularly by vegetarians (both lignans and phytoestrogens) [33, 34, 58], by subjects consuming large amounts of whole-grain products, vegetables and berries, which all are associated with high lignan excretion [33], and by the Japanese consuming traditional Japanese diet (mainly isoflavonic phytoestrogens, due to intake of soy products) [33, 72]. In omnivorous Finnish subjects the excretion of Gen, the specific inhibitor of protein tyrosine kinase, was found to be between

10 and 1,500 nmol/24 h (usually 1-4 times that of Da). When investigating a few Japanese subjects consuming a traditional diet the excretion was very high ranging from 1,250 to 15,500 nmol/24 h (!) (in collaboration with H. Honjo and coworkers), about 1.5 - 3 times higher than that of Da. As mentioned Da shows antiproliferative activity with regard to BC cells [59]. Particularly low excretion of these compounds has been observed in BC patients and in subjects consuming a low-fiber diet, especially a diet low in whole-grain products and beans [23, 24, 49, 64, and unpublished results]. Particularly low excretion has been observed in BC patients and in subjects consuming a low-fiber diet, particularly a diet low in whole-grain products [33, 34, 58, 73].

It has now been demonstrated that the mammalian lignans Enl and End are formed from precursors, such as the plant lignans matairesinol and secoisolariciresinol, which are consumed and then structurally modified by intestinal bacteria [56]. Eq and O-Dma are most likely formed by intestinal bacterial action from formononetin (For) and Da present in food stuffs like soy products [72, 74]. However, these compounds are also present in cow milk [75] formed from *e.g.* For in clover by intestinal bacteria in the gastrointestinal tract of the cow [55], and may therefore be consumed by human subjects as such. Because of the close association of lignan excretion with fiber intake [21, 33, 44] it is likely that the plant lignans are localized close to the outer fiber-containing layers of the grain containing phytin, polyphenols, enzyme inhibitors and other compounds usually regarded as antinutritional factors [76].

Recently, we suggested that the lignans and isoflavonic phytoestrogens, which all are diphenols, perhaps together with other similar compounds, stimulate SHBG synthesis in the liver and in this way reduce the biological effects of sex hormones [27, 33, 34]. An increase in SHBG results in lowering of %FT and %FE2 and reduction of both the albumin-bound and the free fraction of the sex hormones. This reduces the metabolic clearance rate (MCR) of the steroids and reduces in this way their biological activity.

In Finnish women total fiber intake, total fiber intake/kg body weight and grain fiber intake/kg body weight correlate positively and dietary fat/fiber ratio negatively with urinary excretion of total lignans and isoflavonic phytoestrogens [33, 34]. The excretion of the two diphenolic groups of compounds and also Enl alone in both pre- and postmenopausal Finnish women correlate positively with plasma SHBG and negatively with plasma %FE2 and %FT [33, 34 and unpublished results]. It is well known that oral estrogens, in contrast to parenterally administered ones, markedly stimulate SHBG synthesis [77, 78] and we therefore suggest that these positive associations between urinary lignan and phytoestrogen excretion and SHBG is due to stimulation of SHBG synthesis by these week estrogens entering the portal circulation in very high amounts. This also would explain the higher SHBG values seen in vegetarians [79] including such vegetarians whose diet does not contain low amounts of proteins [34]. High protein diet has been found to lower plasma SHBG [36, 37].

Furthermore urinary Enl excretion in these Finnish women correlates negatively with plasma DHEAS and luteinizing hormone (LH) (unpublished observations). The latter observation has to be evaluated in detail, but it is possible that the effect on sex hormone metabolism of these weakly estrogenic compounds may also be mediated via an effect on the hypothalamic–hypophyseal endocrine system. Plasma DHEAS is low in vegetarians and is negatively associated with the dietary intake of unsaturated fatty acids [35].

DIET, SEX HORMONES AND BREAST CANCER

In an extensive review about 10 years ago Dao concluded that studies of estrogen metabolism in BC has provided only controversial results and that they are inconclusive at best [80]. The results described above indicate clearly that studies on sex hormone metabolism in cancer cannot be carried out without careful dietary evaluation in the subjects studied. It is therefore not surprising that no consensus as to the association between sex hormone changes and BC has been reached, because very few studies include both detailed dietary records and hormonal investigations.

In his recent review Zumoff [81] includes nine hormone-related hypotheses in the discussion on hormones and BC, but none of them was discussed in relation to diet despite the huge amount of epidemiological data suggesting that a Western diet plays an essential role in increasing the BC risk in the Western world.

Because of the extensive literature I will discuss only a few of those hypotheses regarding the association of sex hormone alterations and BC, which seem to be related to diet.

The main change in diet when subjects from developing countries migrate to Western countries is an increase in animal fat and protein and a decrease in intake of complex carbohydrates, particularly whole grain products [10]. This change is identical to what has occurred in Scandinavia in the last 300 years and in fact has been going on in Finland since World War II with a simultaneous increase in the incidence of BC, CC and other Western diseases. I therefore like to discuss particularly the possible role in cancer development of complex carbohydrates like whole grain products and soy beans, cereal fiber and the role of lignans and isoflavonic phytoestrogense and their association with plasma SHBG and the % free sex hormones.

In two case-control [82, 83] and in an epidemiological study [84] it was shown that high fiber and high carbohydrate intake, respectively, decreased the risk of BC. In another case-control study particularly fiber from grains consumed during adolescence reduced the risk both in premenopausal and postmenopausal women [85]. These observations are in agreement with the results of our studies in postmenopausal women in Boston [35] and in premenopausal women in Helsinki [41] showing that the main and in fact only really significant difference between the diet of the BC patients and the omnivorous and vegetarian control women was a low intake of grain products and grain fiber. If the diets of the Boston and Finnish women studied by us are compared, the main difference is also in the grain and grain fiber intake, being much higher in the Helsinki women with a lower risk for BC than the Boston women. This dietary difference caused the mean fecal weights to be higher in the Finnish compared to the Boston women, despite similar mean total fiber intakes. The large fecal bulk affects the enterohepatic circulation of sex hormones, because there is *e.g.* a significant correlation between fecal weight and fecal estrogens. In both countries the fat/fiber ratio was the same in the omnivorous and BC women, but much lower in the vegetarian women, particularly in Boston, because the Finnish vegetarians consumed rather much fatty milk products. The postmenopausal Boston BC women had lower fat intake than the Finnish young vegetarians (!), the protein intake being similar. However, the fat to grain fiber ratio (g/g) was 16.4 in the old Boston BC women and only 10.2 in the young Finnish BC women and the corresponding values for the omnivores were 15.1 and 8.2, respectively. The Boston and Helsinki vegetarians had total fat/grain fiber ratios of 7.1 and 6.3, respectively. Very interesting are also the results of the protein/grain fiber (g/g) ratios in the six groups of women, The vegetarians, omnivores and BC patients in Boston and Helsinki had the following ratios: 7.2, 15.2, 18.1, and 5.4, 7.2 and 8.8, respectively. This shows that these ratios are very high in the omnivorous women and the BC patients in Boston, and also highest in the BC group in Helsinki compared with the other Finnish women mainly due to differences in grain fiber intake.

The fat intake in the BC women both in Boston and Helsinki was intermediate between that of the omnivores and vegetarians in respective city. This may be due to bias, particularly in Helsinki, because of much propaganda in this country about reducing fat intake in order to avoid cancer and other diseases. However, small differences in fat intake will not have any detectable effect on plasma or urinary sex hormone levels (for discussion see [12]), which may to some extent explain the results of a recent prospective study [86] in nurses that failed to show any correlation between high fat consumption and the subsequent development of BC (see also [87, 88]. However, in our opinion, after considering our above-mentioned results, it seems more appropriate to use the fat/total fiber or fat/grain fiber ratio to define the diet of risk groups and controls than to use % fat calories or total fat intake. However, recent prospective studies in our laboratory suggest that

particularly grain products containing all compounds of the grain may be protective and that so-called whole-meal products may be less satisfactory in this sense (see below). Also protein/carbohydrate or particularly protein/total fiber or protein/grain fiber ratio should perhaps be used to define the dietary groups. Using such ratios we have observed that the association of diet to sex hormone metabolism becomes much more obvious. We believe that this is related to the intestinal metabolism of hormones, lignans and isoflavones, which is dependent on the intestinal environment and closely related to our diet and perhaps better described or reflected by these ratios than by expressing the amounts of macronutritients as percentages of total calories or in relation to body weight.

Without doubt it is not fat alone which has the negative effects on overall sex hormone levels, but proteins, fiber and complex carbohydrates seem at least in Western societies to play even more essential roles. As an example of what this concept means is the increasing effect of a high fat and meat and low grain intake both in man and in experimental animals on intestinal β-glucuronidase (see literature in [21, 23]), which theoretically leads to an increase of the reabsorption of estrogens from the intestinal tract [25] and higher plasma estrogen levels [23, 24]. It should also be emphasized that the associations between fiber intake and the excretion of a number of urinary estrogens became statistically significant first when the fiber/kg body weight ratio was used instead of total fiber intake [26]. The fiber/kg ratio may better reflect the intestinal bacterial environment and fiber effects because a small subject has a smaller "internal" volume of the intestines compared to a tall subject.

The diet in Finnish rural areas where BC and CC incidence is low differs from the American one particularly with regard to its relatively high content of complex carbohydrates mainly from whole-grain products and starchy vegetables, the fat content being similar but deriving more from milk products than from meat [89, 90 and own observations]. A significant part of the Finnish milk product consumption consists of fermented milk products. Because of the differences in BC risk in USA and Finland we have postulated that this difference is at least partly due to the great difference in intake of whole-grain fiber-rich products like rye bread and perhaps some other fiber-rich nutritients such as berries. Particularly these foodstuffs increase the excretion of urinary lignans by the Finns and affect simultaneously also otherwise the intestinal milieau. This view was supported by the finding of very low urinary lignan excretion in the BC subjects living in Boston [57] and of lower excretion also in the young BC women in Helsinki [34, 73]. In both BC groups it was likely that the differences were due to low intake of whole-grain products. However, in Helsinki the differences between the omnivorious, vegetarian and BC groups were relatively small, because the grain intake was comparably high in all groups, which is typical for the original Finnish diet. It should be mentioned that the intake of wheat germ and bran do not at all cause increases in urinary lignan excretion in human subjects (own observations), and fiber-free wheat bread products have no or only very small influence on lignan excretion. Only grain products which have been made from milling of whole grain, without separating (and washing) the different components and mixing them again (R. Korpela and H. Adlercreutz, to be published) seem to significantly increase lignan excretion in Finnish women. This is because during modern milling of the grain, trying to eliminate so-called antinutritional factors [76], simultaneously also the diphenolic plant lignans seem to be at least partly eliminated. There are indications that also berries, fruits and various seeds [33, 56, 91] increase lignan excretion. Of some grain products, rye meal seems to result in the highest excretion of lignans in rats, followed in decreasing order by oat, barley and wheat meal [91]. The latter results are difficult to evaluate because no exact details were presented regarding the nature of the meal products consumed by the rats.

Based on an epidemiological study it was recently suggested that consumption of fermented milk products may protect against breast cancer [92]. In a case-control study consumption of fat from milk, cheese and yogurt during adolescence reduced the BC risk both in premenopausal and postmenopausal women [85]. One mechanism by which fermented milk may influence hormone metabolism

is by reduction of the β-glucuronidase-producing bacteria of the intestinal content [93, 94], which theoretically should reduce the enterohepatic circulation of estrogens and increase the fecal route of elimination. The conjugated estrogens excreted in the bile must be deconjugated before the estrogen moiety can be reabsorbed. Milk products have also been found to contain animal lignans and isoflavonic phytoestrogens (75) and even if the concentrations are rather low they add to those produced by the intestinal bacteria from plant precursors.

Our hypothesis has been that high intake of whole-grain products (preferably in combination with reduced fat and moderate protein intake) reduces BC (and CC) risk because such a diet increases fecal bulk and reduces intestinal β-glucuronidase activity and steroid and bile acid enterohepatic circulation and results in increased mammalian lignan production [12, 21]. Later on we also included the isoflavonic phytoestrogens into the original theory [33, 54]. This was due to the finding of very high excretion of isoflavonic phytoestrogens in urine of Japanese men and women consuming a traditional diet [33, 72]. The lignan excretion in the Japanese subjects was low, even lower than we found in the postmenopausal BC patients in Boston. The isoflavones resemble lignans with regard to structure (all are diphenolic). In most correlation studies they show parallel behaviour. In the Finnish women the significances of the positive correlation between the excretion of lignans and isoflavonic phytoestrogens in urine, and plasma SHBG, and the negative correlations with %FE2 and %FT are stronger than the separate correlations for each group of compounds [33]. Recently, our hypothesis with regard to the protective role of these compounds for BC got strong support from studies showing that powdered soy bean chips, both before and after denaturation of protease inhibitors, decrease mammary tumor formation in a rat breast cancer model [95]. Furthermore Gen, found by us in human, chimpanzee and cow urine, may be anticarcinogenic due to its inhibitory effect on protein tyrosine kinase [61–64] and other flavonoids are antiproliferative with regard to BC cells [59]. The postmenopausal BC patients in Boston had the lowest plasma SHBG and highest %FT and %FE2 [35] and the lowest Enl and Eq excretion [57]. The Finnish premenopausal BC subjects had lower SHBG, higher %FT and %FE2 and lower excretion of lignans and isoflavonic phytoestrogens compared to the vegetarians [34]. In many studies low SHBG has been associated with BC (see literature in [35, 96]).

Because of the large differences in grain fiber intake and urinary lignan excretion between postmenopausal women living in Helsinki and Boston we have in preliminary calculations combined the materials of postmenopausal women and found the same highly significant positive correlation between grain fiber intake or Enl excretion and plasma SHBG and negative correlations with plasma %FE2 and FT (unpublished observations) as we found for the young Finnish women [33, 34].

The theory based on the observation that high fat intake increases 16α- and decreases 2-hydroxylation of estrogens leading to biologically more active estrogens also needs some discussion. According to this theory a low rate of 2-hydroxylation and high rate of 16α-hydroxylation leads to a greater risk for BC and endometrial cancer [52, 53, 97–99] because 2-hydroxylated estrogens are biologically less active than 16α-hydroxylated ones. Several earlier studies as well as our own seem to speak against this hypothesis because all low-risk groups, compared to high-risk groups, have relatively more urinary 16α-hydroxylated estrogens, particularly if also the fecal estrogens are included. Women living in low-risk countries consume most of their calories in the form of complex carbohydrates and have lower fat and protein intake, which should lead to low 2-hydroxylation of estrogens [38, 39]. This we could observe in the young premenopausal Finnish women [40, 41] and in the previously investigated Oriental women [23, 42]. The characteristics of the sex hormone pattern in these low-risk Oriental women on a low-fat diet are low plasma levels of E1, E2, A and T and low excretion of E1, E2 and 2-hydroxylated estrogens and relatively high amounts of both 16α- and 16β-hydroxylated estrogens [23, 42] We could also not see any increase in 16α-hydroxylated estrogen metabolites in urine of Finnish premenopuasal women with

BC. In fact slightly higher mean values were seen in the vegetarians, but the differences were not significant [40, 41].

Recently we completed the second part of the Finlandia study dealing with groups of postmenopausal women and found results apparently more in line with those suggesting that high 16α-hydroxylation is a risk factor. A statistically significant (logarithmic) negative correlation between plasma SHBG and urinary 16α-hydroxyestrone ($R = 0.59$, $p < 0.001$) and estriol ($R = 0.49$; $p < 0.01$) was found with the highest values of estriol and lowest SHBG values in the BC and omnivorous women and higher SHBG and lower urinary 16α-hydroxylated estrogens in the vegetarians. In the same material there was a significant positive correlation between urinary total diphenol excretion and plasma SHBG ($R = 0.64$; $p < 0.001$). From our results it appears that the tendency to lower values of 16α-hydroxylated estrogens in urine of the vegetarian and higher in the omnivoric and BC women is probably due to different degrees of fecal elimination of these estrogens as a result of differences in fiber intake and not to increased 16α-hydroxylation of estrogens in BC. However, the evaluation of this very large study is still in progress and the definite results have to await the extensive statistical treatment needed. In these postmenopausal women we found no correlation between plasma SHBG and urinary catecholestrogens but a highly significant positive association between the logarithms of plasma E1S and urinary excretion of 2-hydroxy-E1 ($R = 0.84$; $p < 0.001$). The BC women tended to have both higher plasma E1S and urinary 2-hydroxy-E1, which supports our theory that high E1 and E1S and urinary catecholestrogens may be risk factors of BC. It may be mentioned that high E1S has also been found in EC [100].

With regard to 2-hydroxylated estrogens there is evidence speaking for a role of these steroids and catecholestrogens formed from stilbestrol in hormonal carcinogenesis via microsome-mediated redox cycling and formation of quinones and free radicals [101]. The quinoid structures are prerequisites for the genotoxic effect [102] because they are capable of covalent binding to proteins [103, 104]. The development of renal tumors in Syrian hamsters after estrogen treatment has been postulated to occur via a free radical mechanism [105]. Hydroxylated flavonoids have antagonistic effects on the mutagenic and/or tumorigenic activity of epoxide metabolites of polycyclic aromatic hydrocarbons [106]. Because of similar structure the isoflavones and lignans should also be investigated in this respect.

DIET, HORMONES, LIGNANS AND ISOFLAVONES, AND OTHER WESTERN DISEASES

It is not possible in this connection to discuss at any length the relationships between diet and other Western diseases. Some very large reviews on nutrition and its relationship to cancer have been published [107, 108]. However, I would like to discuss shortly some new results indicating that the above discussion may have some important implications also for other diseases than BC and that obvious hormonal and biochemical connections exist between BC and other Western diseases.

Endometrial cancer

What has been said about diet and estrogen metabolism and BC holds as well for EC, a disease even more clearly estrogen-dependent than breast cancer. An increase in bioavailable estradiol due to lowering of SHBG and increase in reabsorption of biliary estrogens as a result of a Western diet would also promote the growth of endometrial cancer. This cancer type has in addition been found to be associated with other diseases common in the Western world, like hypertension and diabetes. Hypertension has in fact recently been found to be a risk factor also of BC [109].

Prostate cancer

Furthermore, it is known that a low-fat and/or high-fiber diet affects sex hormone metabolism also in men [28–30] by decreasing T and FT. A high level of biologically active androgens probably accelerates the development of PC in the Western world and recently a prospective study in fact seems to indicate that elevated T levels are

associated with increased risk of PC [110]. In epidemiological studies fat and meat show a positive and cereals a negative association with PC mortality [3]. In Japan and some other Asian countries, despite the same incidence of latent small or non-infiltrative prostatic carcinomas, the mortality is low [111–113]. This could at least partly be explained by a diet-related lowering of biologically active androgens as seems to occur in Asian women [24] and in the above-mentioned experimental studies [28–30]. Rotkin (cited from [114]) suggested that the men at risk of developing PC had a "strong overbalance of androgenic components" and observed that fewer patients with prostatic cancer developed gynecomastia and obesity early in life compared to controls. However, also recent observations indicate a possible protective effect of endogenous estrogens [115, 116] and this would suggest that the high levels of isoflavonic phytoestrogens in the traditional diet of Japanese men [33, 68] may also represent a protective factor [33, 54] inhibiting the growth of already existing small cancers [theory originally proposed in 117]. However, other than estrogenic effects of these substances may be more important.

The above-mentioned theory gains support from the recent observations of decreased risk of prostate cancer in Adventist men showing high consumption of beans, lentils, peas and some dried fruits (dietary sources of flavonoids) [118] and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which may have some fiber-like effects in the gut) and tofu [119], a soy bean product. Our own results in Japanese men and women [some results in 72] show a strong positive association between the intake of various soy products and urinary excretion of equol and daidzein, and also a positive correlation with lignan excretion, particularly enterodiol, despite the fact that lignan excretion was low in the Japanese subjects investigated. It was in fact suggested [112], that if new small latent carcinomas are being formed at a constant rate they may either disappear or may enlarge and develop into larger carcinomas in different numbers or at different speeds in different geographical areas. It is suggested that in certain populations dietary factors affect androgen metabolism and biological activity as described above and/or that dietary isoflavones and other phytoestrogens directly influence cancer cell growth slowing the speed of development of these small latent carcinomas. The possible effect of soybean diets on PC may be a parallell to the observation of the inhibitory effect of this diet on breast tumor incidence in experimental animals [95, 120].

Coronary heart disease

Low SHBG has been found to be a risk factor of CHD mortality in a female population during a 12-year follow-up period [121] and is probably a risk factor also in men [122]. In addition, low plasma 5α-DHT seems to be a risk factor of CHD in men [122, 123]. As mentioned previously a high dietary protein/carbohydrate ratio not only suppresses plasma levels of SHBG, but simultaneously inhibits liver 5α-reductase [36–39]. Furthermore, we found significantly higher SHBG and HDL-cholesterol and almost significantly higher 5α-DHT ($p < 0.07$) in joggers compared to the subjects with CHD and a positive association between SHBG and HDL-cholesterol and maximal oxygen uptake in both joggers and healthy men [122]. Plasma SHBG and 5α-dihydrotestosterone concentration correlates positively with HDL-cholesterol and apoliprotein A-I both in healthy middle-aged men and in men with CHD [122, 123]. It is also known that thyroid hormones and estrogens stimulate SHBG synthesis, increases liver 5α-reductase and plasma HDL-cholesterol and apolipoprotein A-I [124, 125]. In population studies HDL-cholesterol and apolipoprotein A-I are inversely related to CHD [126, 127]. Compounds increasing the 5α-/5β-reductase activity ratio in rat liver microsomes lower serum cholesterol and reduces the incidence and severity of atherosclerotic lesions in aortas of cholesterol-fed rabbits [128]. Whether the higher plasma SHBG and 5α-DHT in our physically fit men compared to the subjects with CHD is due to diet or to physical exercise itself cannot be judged at present. The protein/carbohydrate ratio of the diet may be lower in hard-training joggers, which could explain the high SHBG and 5α-DHT levels. This is because aerobic training usually leads to increased proportion of carbohydrates in the diet.

In Finnish men this may mean increased consumption of whole-grain rye products, because about 40 % of the cereals consumed in Finland are rye products [see *e.g.* 129] and rye bread is usually a whole-grain product in this country.

As already mentioned consumption of whole-grain rye bread has recently been found by us to considerably increase animal lignan excretion in urine (R. Korpela, H. Adlercreutz, to be published), and it also seems to stimulate SHBG synthesis (almost statistically significant increase after 2 weeks; $p < 0.07$) as suggested previously [33]. Furthermore, it is of interest that isoflavones, excreted in high amounts in urine in populations having a low CHD risk, like the Japanese men, have hypocholesterolemic effects in rats [130] and that treatment with a soybean-protein diet has remarkable hypocholesterolemic effects in human subjects with type-II hyperliproteinemia [131]. Soybean protein products contain isoflavonic phytoestrogens, but whether the effect observed is due to these compounds or to the plant protein itself, as suggested by the authors, is uncertain. It is interesting to note that it has been suggested that the hypocholesterolemic effects of isoflavones is probably independent of the estrogenic effects [130]. Furthermore, it has been shown that the hypocholesterolemic effect of soy products in human subjects is not due to the content of soybean fiber [132]. It is concluded that very similar associations between diet, SHBG, lignans and isoflavones, as found for BC, seem to exist also with regard to CHD.

Colon cancer

In epidemiological studies a parallelism has been observed between BC and CC [133], but there are also some discrepancies suggesting different etiology [review 86]. However, for none of the Western diseases the etiology is likely to be monofactorial and looking only for the associations with macronutritients may easily lead us to wrong conclusions. There are also some parallelisms between CC and PC [3], and diet, in the majority of the opinions [107,108], seems to be the most important environmental factor in the development also of CC.

CC has also been found to be related to reproductive and hormonal factors [review in 130] and it has been found that increasing parity decreases risk and late age at first live birth increases risk [135, 136] as found also for BC. Women with cancers of the breast and other reproductive sites have an excess of primary colorectal cancer and pregnancy protects against DMH-induced colon cancer in experimental animals [review in 136]. Many colon tumors contain sex hormone receptors [137-140], and they may play a role in the pathogenesis of the disease [141].

The observed discrepancies in parallelism between CC and BC incidence and mortality development in Japan [86] may be due in addition to changed consumption of macronutritients to some micronutritients like plant lignans and isoflavones having a large spectrum of biological activities like anticarcinogenic, antiproliferative, antihormonal or hormonal and antiviral effects, which may play a role also locally in the intestine [21, 142]. The local effects in the intestine may be independent of the formation of the hormonally active substances which seem to alter liver and peripheral sex hormone metabolism. Another factor which may play a role for the discrepancies in parallelism between CC and BC is that a change in the fat content of the diet e.g. in Japan may not parallel a change in the use of soy products, because the soy sauce is mainly used for its content of sodium chloride and other soy products may still be used independently of an increase in fat intake. When leaving the habit to consume a low-fat diet the Japanese seem to still consume rice and they do not get any additional (cereal) fiber needed to compensate for the higher fat intake, because whole-grain bread seems to be almost unknown in Japan. This in our opinion could perhaps explain that the CC incidence in Japan increases more rapidly than the BC incidence [86] because of the absence of cereal fiber but continuous consumption of soybean products and rice.

Furthermore, an increase from 10 to 25 % of the fat calories as has occurred in Japan between 1955 and 1975 [86] may not alter the hormonal pattern as much as the difference we find for urinary and plasma sex hormones when the fat calory intake is

about 20 % compared with that found when it is about 38 % [24, 42]. In our own studies in a rural village outside Kyoto [72] women and men still consume only 20 and 17 % fat calories, respectively.

In most epidemiological studies a relation between fat intake and CC has been observed, but in only few studies an association has been found between CC risk and high protein intake or high energy consumption [143, 144] both leading to low SHBG, despite the fact that fat and protein consumption generally increase in parallel. In one study a high meat/vegetable consumption ratio predisposed for CC [145], a diet, which probably also would affect sex hormone pattern [35].

However, as for BC, a negative association between CC and intake of cereals or nonstarch polysaccharide fiber has been observed in most (but not all) epidemiological studies [review in 146, 147], the case-control studies being less convincing [see 147]. To my knowledge no prospective studies on effect of grain fiber or whole-grain products on CC incidence have been published. Recent studies suggest that the fat/fiber ratio is important also in the pathogenesis of CC because a negative association between CC and dietary fiber was found only in men with low fat consumption [148]. Epidemiological studies in Finland and Denmark point to a protective role of cereal fiber [89, 90, 129, 149], but also other factors like high consumption of fermented milk lowering colonic pH [150, 151] and supplying calcium [152, 153] [review in 154] are most likely partly responsible for the favourable CC incidence in rural Finland. Thus fermented milk may play a role for both BC (lowering effect on intestinal ß-glucuronidase) and CC risk [94, 154, 155]. As indicated above the dietary fat/fiber ratio seems to determine the degree of the enterohepatic circulation of hormonal steroids and may in this way alter the risk of hormone-dependent cancers. In experimental colon carcinogenesis this ratio determines the tumor prevalence and dietary fiber content determines the bile acid concentration and protects against the deleterious effects of fat [156, 157].

Because of the relatively high consumption of whole-grain rye bread in Finland we have been interested in studying whether different cereal products may have different effects on the CC risk factors. From these studies we have now obtained more support for the theory [21] that certain fiber-rich grain products, supplying precursors for mammalian lignan formation perhaps protecting against BC and locally having a favourable influence on intestinal bacterial composition and metabolism and mucosal cell environment, may be protective with respect to CC also by another mechanism. This is because rye bread seem to favourably influence intestinal bile acid metabolism. In a recent experiments we observed that by changing the bread consumption from a wheat fiber-free bread or from a whole-meal fiber-rich (fiber > 9 %) wheat bread to a whole grain rye bread (fiber > 8 %), significant alterations of the biochemical risk factors of CC could be obtained (see below), suggesting that the relatively small dietary change may have positively affected intestinal metabolism. The rye bread made from whole grains, not purified during milling, compared to both the fiber-free and a fiber-rich wheat bread (produced after modern milling of the grain eliminating some fractions, but containing essentially all components) increased considerably the urinary lignan excretion (R. Korpela & H. Adlercreutz, to be published). Compared to the control period no change (whole-meal) or a decrease (wheat, fiber free) was observed for the other breads. As mentioned above we have shown that lignan excretion is low in women with BC [21, 34, 57], most likely due to low intake of whole-grain bread. Furthermore, it has been shown that autohydrolyzed lignin, which is a polymer with similar basic structure as the diphenolic lignans, protects against experimental colon adenocarcinoma in rats [158]. Lignin is also known to bind deoxycholic acid very well compared to other types of fiber [159]. The effect of rye bread (200 – 300 g per day, no other cereal products consumed) on intestinal bile acid metabolism was remarkable because it considerable decreased the total free bile acid, and total and free secondary bile acid concentrations and the ratio of secondary to primary bile acids in feces (J. T. Korpela, H. Adlercreutz & R. Korpela, to be published) leaving, however, the LCA/DCA ratio unchanged. This ratio increased with consumption of the fiber-free

wheat bread. The reason for the decrease in free bile acids was a huge increase in the concentration of saponifiable (esterified) bile acids to a mean of about 46 % of total bile acids. These esters have been found to form a high proportion of the bile acids in feces in vegetarians (up to 80 %) but occur in very low amounts in CC patients (mean about 10 % of total bile acids) [160]. According to our theory the saponifiable (esterified) bile acids may not be cocarcinogenic or comutagenic as found for the aminoconjugates of these acids [16]. The reason for this may be that they are nonpolar and therefore less water-soluble which may be advantageous [152]. With the other types of bread practically no change of bile acid pattern occurred, or if any, it was in the opposite direction, particularly with respect to the fiber-free wheat bread. This is in agreement with a previous study showing no change in fecal bile acid excretion after consumption of a wholemeal bread compared to "white bread" [161]. In this connection it is of interest to note that during wartime the milling of flour resulted in much higher fiber, and possibly lignan precursor contents, which seems to have resulted in a modest decrease in colon cancer mortality [162]. These results would imply that by a simple change of the bread consumption to a daily intake of 200 – 300 g of whole-grain rye bread (or some other grain?), containing all the components of the cereal, the risk for both BC and CC could at least theoretically be reduced. Interestingly recent associations have been found both between BC [163] and CHD [164], and adenomatous polypes in colon, which are regarded as the first stage of some CC tumors.

Our results with respect to fecal bile acid metabolism are not in disagreement with the original theory of Hill *et al.* [13], but extend the theory to include the degree of "esterification" (the saponifiable bile acids have not yet been characterized). It is still most likely that the concentration of free secondary bile acids is an important factor determining the CC risk [15–20, 165, 166].

CONCLUSIONS

In conclusion, it seems that a Western diet with high fat and protein intake and low intake of fiber, complex carbohydrates and whole-grain products is associated with high plasma sex hormone levels and low SHBG, 5α-DHT, high %FT and %FE2, high urinary and low fecal excretion of estrogens, high urinary catecholestrogens excretion and 2-hydroxy-E1/4-hydroxy-E1 ratio, and low urinary excretion of lignans and isoflavonic phytoestrogens. These compounds apparently are protective with regard to cancer by many different mechanism. With respect to plasma hormones (except 5α-DHT), urinary lignans and equol we found this pattern in the postmenopausal BC women in Boston. Furthermore such a diet leads to unfavourable plasma lipid levels and intestinal bile acid metabolism most likely increasing the risk for both CHD and CC. In the study in Finland, where the BC and CC incidences are much lower than in USA, the hormonal pattern in the young BC patients was very similar to that of the control omnivorous and vegetarian women (33, 34), probably because of the relatively high intake of grain products [41] in all groups studied, but mean grain intake was still lowest in the BC group. The situation may be different in premenopausal compared to postmenopausal women, but still nothing speaks against the theory that diet is an important BC risk factor. This seems to be the fact particularly in the postmenopausal women, but probably and perhaps to a lesser degree, also in young women. All dietary components seem to have their specific role(s) in influencing sex hormone metabolism as described above and in this way a wrong diet may influence the development of BC and other sex hormone-dependent cancers in the promotional stage of the disease. More work is still needed, but already now it seems that the above-mentioned studies showing very distinct associations between diet and sex hormones and SHBG and diet and fecal bile acid pattern fit rather well with the view of the epidemiologists, that Western diet is the main factor causing the high incidence of hormone dependent cancers and CC in the Western world. Furthermore, many significant biochemical and hormonal connections between BC and other Western diseases, like CHD, exist, indicating that the same type of diet partly by the same mechanisms may be responsible for several of these diseases.

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Japanese Unexamined Patent Publication (Kokai) No. 62(1987)-126186
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Inventors: Takuo Kosuge et al.
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1. Title of the Invention

PROCESS FOR PRODUCING ISOFLAVONE DERIVATIVES

2. Scope of Claims for Patent

A process for producing isoflavone derivatives, characterized by bringing a soybean extract, either as such or after removing a solvent by distillation, into contact with a synthetic adsorbent resin to adsorb isoflavone derivatives on the resin, and subsequently eluting the isoflavone derivatives with an organic solvent or a mixed solvent composed of an organic solvent and water from the synthetic resin to obtain the isoflavone derivatives.

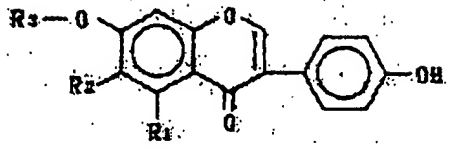
3. Detailed Description of the Invention

[Field of Utilization in Industry]

The present invention relates to a production process of isoflavone derivatives, which is simple and inexpensive.

[Prior Art and Problems]

Soybeans as leguminous (Leguminosae) plants have long been cultivated as valuable vegetable protein sources and are used as raw materials for various foods. Soybeans contain proteins, sugar, and vitamins and, in addition, isoflavone derivatives such as daidzin, glycitin, genistin, daidzein, and genistein represented by the following formula, and are known to have many pharmacological actions including that daidzein has papaverine-like analgesic action in mouse harvested small intestine [Yakugaku Zasshi, 97. 103 (1977)]:



	R_1	R_2	R_3
Daidzin	H	H	Glucose
Glycitin	H	OCH_3	Glucose
Genistin	OH	H	Glucose
Daidzein	H	H	H
Genistein	OH	H	H

Therefore, when these isoflavone derivatives are provided as pharmaceuticals in the future, that the isoflavone derivatives can be obtained at low cost and in large amounts is an important factor.

In order to obtain isoflavone derivatives, methods have hitherto been adopted in which an extract of soybeans with an organic solvent or a hydrous organic solvent is purified by column chromatography using alumina, silica gel or the like as an adsorbent.

When these methods are applied to purification in a large amount on a commercial scale, however, disadvantages such as a significant increase in burden on the adsorbent and complicated operation due to the expansion of the scale occur. Therefore, isoflavone derivatives can be obtained only in a small amount and, further, are expensive. Further, purification of isoflavone derivatives on a commercial scale is hard to carry out.

[Means for Solving the Problems]

The present inventors have made extensive and intensive studies on a process for producing isoflavone derivatives at low cost and in a large amount. As a result, it was found that isoflavone derivatives can be obtained at low cost and in a large amount by bringing a soybean extract, either as such or after removing a solvent by distillation, into contact with a synthetic

adsorbent resin to adsorb isoflavone derivatives on the resin, and subsequently eluting the isoflavone derivatives with an organic solvent or a mixed solvent composed of an organic solvent and water from the synthetic adsorbent resin to obtain the isoflavone derivatives, which has led to the completion of the present invention.

The present invention will be described in more detail.

In obtaining an extract of soybeans, seeds of Glycine max Merrill may be used as soybeans for extraction. Water, an organic solvent, or a mixed solvent composed of water and an organic solvent may be mentioned as the solvent for the extraction. The extraction is conducted in a temperature range of at room temperature to the boiling point of the extraction solvent.

At the present time, about 180000 tons per year of soybeans are consumed, across the country, in the production of *miso* (soybean paste). In this case, most of broth produced by boiling in water is discarded as a waste liquid. The extract of the soybean referred to in the present invention embraces this waste liquid. Accordingly, the utilization of soybean extract, produced during the production of *miso*, which has hitherto not been used, can realize the production of isoflavone derivatives at lower cost.

Next, the extract either as such or after the removal of the solvent by distillation is brought into contact with a synthetic adsorbent resin to adsorb the isoflavone derivatives on the synthetic adsorbent resin. When there is a variation in acidity or basicity in the extract, the extract is preferably kept at a pH value of about 3.5 to 5.0 by properly adding a suitable acidifying agent or alkalifying agent from the viewpoint of improving the adsorption of the isoflavone derivatives on the synthetic adsorbent resin. Acidifying agents usable herein include acetic acid and hydrochloric acid. Alkalifying agents usable herein include sodium hydrogencarbonate and sodium hydroxide.

When an aqueous solvent is used as the extraction solvent at the time of the extraction, previously removing the used aqueous solvent from the extract by distillation is preferred from the

viewpoint of improving the adsorption of isoflavone derivatives on the synthetic adsorbent resin.

Specific examples of synthetic adsorbent resins include "DIAION HP resin" (manufactured by Mitsubishi Kasei Corp.), "Amberlite XAD resin" (manufactured by Rohm & Haas), and "Duolite S resin" (manufactured by Diamond Shamrock).

The contact of the extract with the synthetic adsorbent resin may be carried out by either a batch method or a column method. In the case of the batch method, a conventional method may be used. For example, a method may be adopted in which a synthetic adsorbent resin is placed in a suitable vessel and stirring is properly carried out. In the case of the column method, a conventional method may be used, and the elution rate may be properly selected by taking into consideration various conditions such as the size of the column and the solvent used for elution. Since both the above batch method and column method utilize physical adsorption, preferably, the temperature is approximately room temperature.

Next, the isoflavone derivatives adsorbed on the synthetic adsorbent resin are eluted with an organic solvent or a mixed solvent composed of an organic solvent and water. In this case, any of the batch and column methods may be used, and the solvent for elution may be an organic solvent or a mixed solvent composed of an organic solvent and water. The kind, concentration, and amount of the extraction solvent are properly selected by taking into consideration various conditions such as which of the column or batch methods is used, or the kind and amount of the synthetic adsorbent resin used.

Specific examples of organic solvents include methanol, ethanol, isopropanol, and acetone. Preferred are alcohols, and more preferred are ethanol and the like.

When the eluate obtained by the batch method, or the eluate obtained by the column method contains a plurality of components, these components can be purified and isolated by conventional separation/purification methods, for example, countercurrent

distribution, recrystallization, or column chromatography.

The synthetic adsorbent resin used in the production process of the isoflavone derivatives according to the present invention can be repeatedly used by washing/regeneration with a suitable organic solvent, for example, alcohol and acetone organic solvents or alkaline chemicals, for example, sodium hydroxide and potassium hydroxide, and thus is very cost effective.

[Examples]

Next, the present invention will be described in more detail with reference to the following Examples. However, the present invention is not limited to these Examples.

Example 1

Soybean broth (3 liters) produced during the production of *miso* (soybean paste) was adjusted to pH 4.0 by the addition of acetic acid and was then filtered through a cotton stopper to obtain a filtrate. Next, washing with 600 ml of methanol and 600 ml of water was carried out. The filtrate was added to a resin column packed with 500 ml of an activated styrene-divinylbenzene polymer resin (DIAION HP-20, manufactured by Mitsubishi Kasei Corp.) and was passed through the resin column at a rate of 50 ml/min. Subsequently, 600 ml of water and, further, a 20% aqueous methanol solution were added to the column for washing. After washing, 1.8 liters of ethanol was passed through the resin column at an elution rate of 30 ml/min to obtain a solution containing daidzin, glycitin, genistin, daidzein, and genistein.

Example 2

An 80% aqueous ethanol solution was added to 20 g of commercially available defatted soybeans. The mixture was refluxed on a water bath for 4 hr, was cooled, and then filtered through filter paper. Water (150 ml) was added to 50 ml of the extract thus obtained, and the diluted extract was adjusted to pH 4.0 by the addition of acetic acid. This liquid was added to a resin column packed with 20 ml of a styrene-divinylbenzene polymer resin (DIAION HP-20, manufactured by Mitsubishi Kasei Corp.) activated by washing with 100 ml of methanol and 100 ml of water and was

passed through the resin column at a rate of 1 ml/min. Subsequently, 100 ml of ethanol was passed through the column at an elution rate of 5 ml/min to obtain a solution containing daidzin, glycitin, genistin, daidzein, and genistein.

Example 3

A styrene-divinylbenzene polymer resin (200 ml) (DIAION HP-20, manufactured by Mitsubishi Kasei Corp.) activated by 400 ml of methanol and 400 ml of water was added to a solution provided by adjusting soybean broth (500 ml) to pH 4.0 by the addition of acetic acid, and the mixture was allowed to stand at room temperature for 2 hr with occasional stirring. This solution was filtered through Kiriyama Roht funnel, and the residual liquid was transferred to a column. Water (400 ml) was added to the column for washing, and 2 liters of a 70% aqueous methanol solution was then passed through the column at an elution rate of 50 ml/min to obtain a brown solution containing puerarin and daidzin. Next, 2.5 liters of methanol was passed through the column to obtain a brown solution containing daidzein.

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Translator's Note: The Japanese text is partially indefinite. Our translation was prepared to convey the meaning thereof.

in newborn babies support an essential role for n-3 fatty acids in retinal development.[5]

The DHA content of erythrocytes is small compared with that of grey-matter, but the fatty acid composition of the erythrocyte membranes may indicate the fatty acid status of neural and perhaps other membranes. During the period of most rapid DHA accumulation in the developing rat, diet-induced changes in neural DHA are reflected in red blood cell DHA.[6]

Dietary n-3 fatty acids can also modify endogenous prostaglandin production and perhaps by this means influence uterine prostaglandins and gestation time. In the Faroe Islands, where birthweights are amongst the highest in the world with long gestation periods and rapid fetal growth, the intake of marine fat rich in n-3 fatty acids is high and erythrocyte DHA values in pregnant women were found to be almost twice those in normal individuals in other countries.[7]

The lower erythrocyte DHA found in patients on epoetin could be due to an increased requirement for this n-3 fatty acid as a result of increased red cell production, and this implies a deficiency of or a rate-limited production of DHA. However, plasma DHA values were not low, which raises the possibility of a defect of incorporation of this fatty acid into the membrane in patients on haemodialysis.

A low membrane DHA probably has little effect on red cell function and may be of minor importance in adults, although it is of interest that visual hallucinations have been described in patients on epoetin.[8] Unlike the adult, the fetus requires DHA in quantity for its developing nervous system, and haemodialysed patients do occasionally become pregnant. For reasons not fully understood, pregnancy in uraemia is associated with a high risk of premature labour and retarded fetal growth.[9] A lack of DHA would be detrimental to the fetus, and our results indicate that in a uraemic pregnant woman on haemodialysis, low quantities of membrane DHA could be one of the hazards to which the fetus is exposed. Because epoetin gives rise to even lower membrane DHA content, its use could increase the risk to the fetus: n-3 fatty acid dietary supplements are indicated.

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Dietary phyto-oestrogens and the menopause in Japan

SIR,—Lock, in an article on the menopause,[1] has discussed differences between Japanese women and women in western societies. Japanese women have a much lower frequency of hot flushes than women in Canada. Lock concluded that "cultural indifference to the hot flush in Japan" was unlikely to account fully for these findings.

Recently our Helsinki group studied, in collaboration with Japanese scientists, the diet and phyto-oestrogen excretion in Japanese women and men, and in a few children.[2] The women's mean age was 50·4 (SD 18·0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. We studied a group of three men, three women, and three children living in Kyoto and consuming the traditional diet, and in this group we measured the isoflavonoid genistein.[2] We found a very high excretion of phyto-oestrogens in urine. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15·5 μmol per 24 h in a man) and two other isoflavonoids, daidzein and equol (table). All these compounds bind to oestrogen receptors and have weak oestrogenic activity.[3] The excretion of the isoflavonoids in urine of the Japanese women was much higher than in American and Finnish women (table) (ref 4 and unpublished data) and as high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than those of endogenous oestrogens in normal omnivorous women consuming a western or oriental diet (table).

URINARY EXCRETION OF ISOFLAVONOID PHYTO-OESTROGENS AND ENDOGENOUS OESTROGENS IN JAPANESE OR ORIENTAL WOMEN, AND IN AMERICAN AND FINNISH OMNIVOROUS WOMEN

Urinary isoflavonoid or oestrogen	Japanese/ Oriental	American	Finnish
Genistein	3440 (n = 3)*	..	32·1 (n = 12)
Daidzein	2600 (n = 10)*	216 (n = 21)	40·5 (n = 12)
Equol	2600 (n = 10)*	62·8 (n = 21)	44·2 (n = 12)
Oestrone (postmenopausal)	4·48 (n = 9)†	..	4·48 (n = 10)
Oestradiol (postmenopausal)	0·76 (n = 9)†	..	0·94 (n = 10)
Oestriol (postmenopausal)	4·48 (n = 9)†	..	4·44 (n = 10)

All assays by gas chromatography/mass spectrometry in selected ion-monitoring mode with deuterated internal standards.[4,5] Women collected two to four 72 h urine samples 3–6 months apart and values are thus means of urinary excretion in individual subjects over 6–12 days. Results as geometric means in nmol/24 h.
*Values from ref 2.
†Oriental postmenopausal women (recent immigrants to Hawaii). Same women as in ref 7, but oestrogens measured by new technique.[6]

The excretion of the isoflavonoids in urine was associated with intake of soy products such as *tofu, miso, aburage, atuage, koridofu,* soybeans, and boiled beans. All isoflavonoids are weak oestrogens and such high amounts could have biological effects, especially in postmenopausal women with low oestrogen levels. High levels of isoflavonoid phyto-oestrogens may partly explain why hot flushes and other menopausal symptoms are so infrequent in Japanese women.

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(2)

D4

Japanese Unexamined Patent Publication (Kokai) No. 61(1986)-246124
Publication Date: November 1, 1986
Application No. 60(1985)-89770
Application Date: April 24, 1985
Inventors: Hiroshi Ogawara et al.
Applicants: Yamanouchi Pharmaceutical Co., Ltd. and Hiroshi Ogawara

Specification

1. Title of the Invention

CARCINOSTATIC AGENT

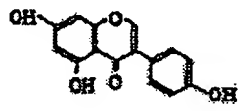
2. Scope of Claim for Patent

An carcinostatic agent comprising 5,7,4'-trihydroxyisoflavone (genistein) as an active component.

3. Detailed Description of the Invention

(Field of Utilization in Industry)

The present invention relates to a carcinostatic agent comprising, as an active component, 5,7,4'-trihydroxyisoflavone (general name: genistein) represented by formula



(Prior Art)

Genistein is a conventional compound described in Journal of the Chemical Society, p. 3447, 1951. This literature reports that genistein is a compound isolated from a certain kind of clover (Trifolium subterraneum L.) and has low estrogenic activity. This literature, however, does not report any anticancer action of genistein.

(Operation and Effect of the Invention)

The present inventors have found a substance having anticancer action in a fermentation product of a microorganism belonging to the genus Pseudomonas separated from soil, and, as a

result of further search, it was found that this substance is genistein. This has led to the completion of the present invention.

The anticancer action, toxicity and the like of the compound according to the present invention will be described.

① Tumor cell growth inhibitory activity and DNA synthesis inhibitory activity

The anticancer action of genistein was investigated by the following experimental tumor cell growth inhibition and DNA synthesis inhibition tests.

(i) Test on growth inhibition against rat transformed cells (RSV-3Y1 cells) by Rous sarcoma virus

(ii) Test on growth inhibition against human epithelial cancer cells (A 431 cells)

(iii) Test on growth inhibition against rat transformed cells (SV 40-3Y1 cells) by SV 40 virus

(iv) Test on DNA synthesis inhibition against mouse mastocytoma (P815 cells)

(v) Test on DNA synthesis inhibition against mouse thymus (EL-4 cells)

Testing methods and results

Methods for the above tests (i), (ii) and (iii) are as follows.

(i) RSV-3Y1 cells, (ii) A431 cells, or (iii) SV 40-3Y1 cells were cultured in a Dulbecco's MEM (manufactured by Nippon Suisan Kaisha, Ltd.) medium containing 2% fetal calf serum (manufactured by Gibco) and genistein at various concentrations. The concentration of genistein was on four levels of 0 (zero), 1 μg/ml, 3 μg/ml, and 10 μg/ml. One day, two days, three days, and four days after that, the vial cell count per dish was measured with trypan blue. The results are shown in Figs. 1 (a) and (b).

As shown in Fig. 1, genistein exhibited cell growth inhibitory action when added in an amount of about 1 to 3 μg/ml, and exhibited significant growth inhibitory action when added in an amount of 10 μg/ml.

Methods for the above tests (iv) and (v) are as follows.

(iv) P815 cells or (v) EL-4 cells were suspended in an RPMI 1640 medium (manufactured by Nippon Suisan Kaisha, Ltd.) containing 2% fetal calf serum inactivated at 56°C for 30 min (manufactured by Flow Laboratories) and 80 μg/ml gentamicin (manufactured by ESSEX NIPPON K.K.) to give a final cell concentration of 2×10^5 cells/ml. This cell suspension was placed in a 96 well flat-bottom microplate (manufactured by Sumitomo Bakelite Co., Ltd.) in an amount of 200 μl/well, and genistein was added to give final concentrations of 0 (zero), 1 μg/ml, 3 μg/ml, and 10 μg/ml. This plate was cultured in a 5% CO_2 incubator (37°C) for 24 hr. Thereafter, 0.1 μCi/well of [^{3}H] thymidine (manufactured by Amersham Japan Ltd.) was added thereto, followed by culture for additional 18 hr. For each well, the cells were collected on a glass fiber filter (Whatman GF/C). The filter was dried and was then placed in a scintillation vial. A toluene scintillator was added thereto, and [^{3}H] thymidine uptake was measured with a liquid scintillation counter. The results are shown in Fig. 2.

As can be seen from Fig. 2, when 3 μg/ml of genistein is present in the culture, in P815 cells, thymidine uptake could be inhibited by about 50%. In a concentration of 10 μg/ml, for both P815 cells and EL-4 cells, the uptake of thymidine could be fully inhibited.

② Inhibitory action against tyrosine specific phosphorylated enzyme activity

Inhibitory action of genistein against various enzyme activities was determined for the following three (a to c) tyrosine specific protein kinases, two (d and e) serines, threonine protein kinase, and other enzymes (f to h).

(a) Rous sarcoma virus-derived (Src gene $pp60^{src}$) tyrosine specific phosphorylated enzyme

(b) Human epithelial cancer cell growth factor receptor (EGF

receptor, A431 cells) tyrosine specific phosphorylated enzyme

(c) Feline sarcoma virus-derived (fes gene, $pp110^{fes}$) tyrosine specific phosphorylated enzyme

(d) c-AMP-dependent protein kinase

(e) phosphorylase kinase

(f) phosphodiesterase

(g) Na^+, K^+ -ATPase

(h) 5'-nucleotidase

Among them, (a) to (c) are cancer gene-derived tyrosin specific phosphorylated enzymes, and (d) and (e) are serine and threonine protein kinases.

Methods for measuring the enzyme activity inhibitory action of genistein and the results will be described.

Measuring methods

(a) Method for measuring Rous sarcoma virus-derived (Src gene $pp60^{src}$) tyrosine-specific phosphorylated enzyme activity (see M.S. Collette, R.L. Ericson: Proceeding of the National Academy of Sciences of the USA, Vol. 75, p.2021-2024, 1978)

3Y1 cells (rat fetus kidney-derived fibroblasts) transformed with Rous sarcoma virus (RSV) are cultured. After washing, RIPA buffer [0.5% NP40, 0.1% sodium deoxycholate, 50 mM Tris-hydrochloric acid (Tris-HCl) pH 7.2, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 0.15 M NaCl] is added thereto, and the mixture is allowed to stand at 0°C for 30 min for solubilization. The culture is then centrifuged at 100,000 x g for 20 min. Antiserum obtained from a rabbit bearing a cancer induced by inoculation with RSV is added to the resultant supernatant, followed by incubation at 0°C for 30 min to one hr to react $pp60^{src}$ with an antibody. The immune complex is collected by mixing with protein A-Sepharose-4B (manufactured by Pharmacia) and is then washed with an RIPA buffer. The pp 60^{src}-antibody-protein A-Sepharose-4B complex reacted in 20 mM Pipes-NaOH pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 10 μM [γ-^{32}P] ATP (2 mCi/mmol) at 30°C for 5 min to conduct a protein kinase reaction. Thereafter, an SDS-containing reaction-stopping solution is added

and the reaction solution is boiled for 3 min to stop the reaction. The reaction solution was electrophoresed on 8% SDS-polyacrylamide gel. After autoradiography, the taken-off $pp60^{src}$ was measured for radioactivity with a liquid scintillation counter to quantitatively determine the phosphorylation reaction.

(b) Method for measuring human epithelial cancer cell growth factor receptor (EGF receptor, A431 cells) tyrosine specific phosphorylated enzyme activity (see S. Cohen, G. Carpenter, L. King; Journal of Biological Chemistry, Vol. 255, p. 4834-4842, 1980)

A cell membrane adjusted from human epithelial cancer cells (A431 cells) known to contain a large amount of EGF receptor was used as an enzyme source. A reaction solution containing, in 50 µl, 20 mM Pipes-NaOH pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, 10 µM [γ-^{32}P] ATP (2 mCi/mmo), A431 cell cell membrane (amount of protein: 10 µg) and genistein is allowed to react for 5 min. The reaction is then stopped, and the reaction solution is analyzed by electrophoresis on 8% polyacrylamide gel-autoradiography to examine the EGF receptor having a molecular weight of 170000 for phosphorylation. Further, the EGF receptor was taken off and was measured for radioactivity with a liquid scintillation counter to quantitatively determine the level of phosphorylation.

Method for adjusting cell membrane from A431 cells

A431 cells cultured in a Dulbecco's MEM (manufactured by Nippon Suisan Kaisha, Ltd.) medium containing 7% fetal calf serum (manufactured by Gibco) were collected, and cell membrane vesicles were adjusted according to the method by Cohen et al. (see Stanley Cohen, Hiroshi Ushiro, Christa Stoscheck, Michael Chinkers: Journal of Biological Chemistry, Vol. 257, p. 1523-1531 1982).

(c) Method for measuring feline sarcoma virus-derived (fes gene, $pp110^{fes}$) tyrosine specific phosphorylated enzyme activity (see R.A. Feldman, T. Hanafusa, H. Hanafusa; Cell Vol. 22, p757-765, 1980)

$pp110^{fes}$ was immunoprecipitated in the same manner as in

$pp60^{src}$, except that rat 3Y1 cells transformed with feline sarcoma virus and serum of Fisher rat bearing a cancer induced by inoculation of the cells were used. The $pp110^{fes}$ was then measured for protein kinase activity.

(d) Method for measuring c-AMP-dependent protein kinase activity

c-AMP-dependent protein kinase (amount of protein: 4 μg) (manufactured by Sigma) adjusted from rabbit muscle reacted in 50 μl of a reaction solution containing 50 mM Hepes-NaOH pH 7.5, 10 mM $MgCl_2$, 4 μM [γ-^{32}P] ATP (2 mCi/mmol), 6 mg/ml histone type IIA (manufactured by Sigma), 10 μM c-AMP and genistein at 30°C for 5 min. Whatman filter paper P81 (2 × 2 cm) was spotted with the reaction solution. The filter paper was washed four times with 50 mM NaCl each for 5 min and was then washed with acetone for 5 min, followed by the measurement of radioactivity with a liquid scintillation counter.

(e) Method for measuring phosphorylase kinase activity

A reaction solution (50 μl) containing 40 mM Tris-hydrochloric acid (Tris-HCl) pH 7.4, 100 μM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 10 μM [γ-^{32}P] ATP (2 mCi/mmol), 10 μg phosphorylase-b (manufactured by Sigma), rabbit muscle phosphorylase kinase (amount of protein: 2 μg) (manufactured by Sigma), and genistein was allowed to react at 30°C for 5 min. An SDS-containing reaction stopping solution was then added thereto, and the mixture was boiled at 100°C for 2 min to stop the reaction. The phosphorylation of phosphorylase b was quantitatively determined by subjecting the reaction solution to 8% SDS-polyacrylamide gel electrophoresis-autoradiography, taking off phosphorylase b, and then measuring phosphorylase b with a liquid scintillation counter.

(f) Measurement of phosphodiesterase activity

A reaction solution (50 μl) containing 50 mM Tris-hydrochloric acid (Trs-HCl) pH 7.5, 8 mM $MgCl_2$, 0.8 mM EDTA, 0.02 mM DTT, 5 mM c-AMP (manufactured by Sigma), bovine heart

phosphodiesterase (amount of protein: 10 μg) (manufactured by Sigma) and genistein is allowed to react at 37°C for 30 min.

10% TCA (50 μl) is added to stop the reaction. The reaction solution is centrifuged at 5,000 rpm for 10 min. The supernatant (90 μl) thus obtained is used for quantitative determination of phosphorus. For a color reaction of phosphorus, 50 μl of a 5 N aqueous sulfuric acid solution containing 5 μl of 1% Triton X-100, 350 μl of purified water, and 2.5% ammonium molybdate was added to the supernatant, and the mixture was allowed to stand for 20 min, followed by measurement of absorbance at 660 nm for quantitative determination.

(g) Method for measuring Na^+, K^+-ATPase activity

A reaction solution (50 μl) containing 50 mM Tris-hydrochloric acid (Tris-HCl) pH 7.5, 60 mM NaCl, 25 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 3 mM ATP, Na^+,K^+-ATPase (amount of protein: 560 ng) adjusted from canine kidney, and genistein was allowed to react at 37°C for 30 min. Thereafter, in the same manner as in phosphodiesterase, phosphorus produced as a result of the reaction was quantitatively determined.

Preparation of Na^+, K^+-ATPase

Na^+, K^+-ATPase was prepared according to the method by Kawamura et al. (see Kawamura, Ota, Nagano: Journal of Biochemistry, Vol. 87, p.1327-1333, 1980) by breaking canine kidney outer medulla with polytron (manufactured by Kinematica) in a buffer containing 50 mM imidazole pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM ATP and then conducting ultracentrifugation to obtain a microsome fraction which was then extracted with SDS.

(h) Method for measuring 5'-nucleotidase activity

A reaction solution (50 μl) containing 55 mM Tris-hydrochloric acid (Tris-HCl) pH 8.5, 5.5 mM $NaCl_2$, 1.1 mM ATP, 10 mM sodium potassium tartrate, 5'-nucleotidase (snake venom) (manufactured by Sigma), and genistein was allowed to react at 37°C for 3 min. Thereafter, in the same manner as in phosphodiesterase,

phosphoric acid as the reaction product was quantitatively determined.

Results

Activity inhibitory action against each enzyme of genistein

Enzyme system	ID_{50} (μg/ml)
(a) $pp60^{src}$ protein kinase	0.8
(b) EGF receptor protein kinase	0.7
(c) $pp110^{fes}$ protein kinase	6.5
(d) c-AMP-dependent protein kinase	> 100
(e) Phosphorylase kinase	> 100
(f) Phosphodiesterase	> 100
(g) Na^+, K^+ -ATPase	> 100
(h) 5'-Nucleotidase	> 100

ID_{50}: 50% inhibition

As is apparent from the above results, genistein specifically inhibits cancer gene-derived tyrosine specific phosphorylated enzyme activity.

The tyrosine specific phosphorylated enzyme is considered to participate in growth of cancer cells. Therefore, the demonstration of the enzyme activity specific inhibitory action supports the anticancer action of genistein.

③ Genistein was intraperitoneally injected into C57BL/6 mice to examine acute toxicity. As a result, LD_{50} was not less than 500 mg/kg.

The results of the tumor cell growth inhibitory action test, the DNA inhibitory action test, and the tyrosine specific phosphorylated enzyme activity inhibitory action test show that genistein has excellent anticancer action and, in addition, has low acute toxicity and thus is useful as carcinostatic agents for the therapy of cancers of humans and animals, the therapy of diseases caused by metastasis of cancers, and the prophylaxis of recurrence.

Genistein is clinically administered as an active component generally at a dose of 200 to 1,000 mg per adult per day. This dose is administered at one time or as divided doses of two to four times. The dose may be properly varied depending upon individual cases such as condition and age of the patient.

Genistein may be used solely for therapy or alternatively may be used in combination with other chemotherapeutic agents or immunotherapeutic agents. Chemotherapeutic agents usable in combination with genistein include cyclophosphamide, vinblastine, vincristine, adriamycin, 6-mercaptopurine, 5-fluorouracil, mitomycin C, bleomycin, aclacinomycin, neocarzinostatin, cytosine arabinoside, cisplatin, actinomycin D, and nitrosourea agents. Immunotherapeutic agents usable herein include, for example, krestin, BCG, picibanil, lentinan, interferon, and interleukin. When genistein is used in combination with these agents, the dose is suitably such that the ratio of genistein to the agent used in combination with genistein is 1 : about 0.001 to 10.

Genistein may be administered in dosage forms, i.e., oral preparations (tablets, capsules, or liquid preparations) or parenterals (rectal administration preparations, injection preparations, or pellets). These preparations are prepared as compositions into which any conventional preparation carrier or excipient has been formulated by any conventional method. In this case, the carrier or excipient used may be commonly used one. For example, in the case of tablets, water, glucose, lactose, gum arabic, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, gelatin, colloidal silica, potato starch, and urea can be utilized. In the case of liquid preparations, aqueous or oleaginous suspensions, solutions, syrup, and elixirs may be used. They may be prepared by a conventional method. For rectal administration, the preparation is provided as a composition for suppositories. In this case, the base may be commonly used one, and examples thereof include polyethylene glycol, lanolin, cacao butter, and Witepsol (registered trademark) (Dynamit Nobel).

4. Brief Description of the Drawings

(1) Figs. 1 (a), 1 (b) and 1 (c) are diagrams showing growth inhibitory activity of genistein against RSV-3Y1 cells, A 431 cells, and SV40-3Y1 cells; and

(2) Fig. 2 is a diagram showing DNA synthesis inhibitory activity of genistein against P815 and EL-4 cells.

FIG. 1 (a)

GROWTH INHIBITORY ACTIVITY OF
GENISTEIN AGAINST RSV-3Y1 CELLS

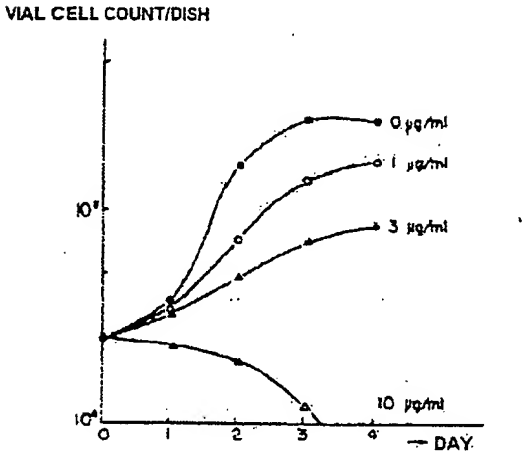


FIG. 1 (b)

GROWTH INHIBITORY ACTIVITY OF
GENISTEIN AGAINST A431 CELLS

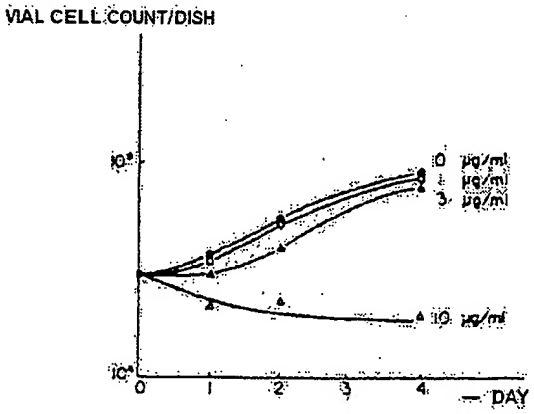
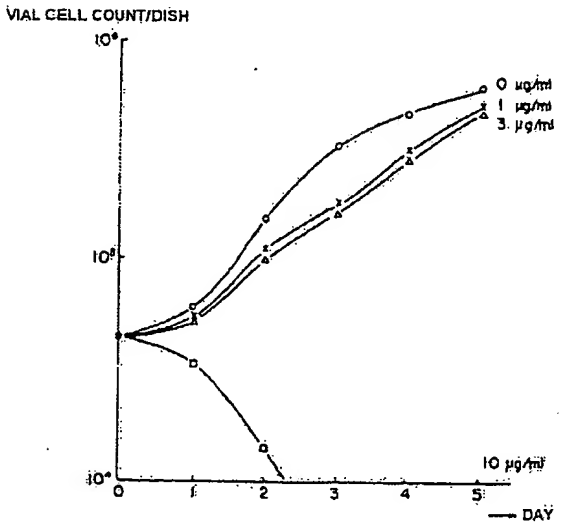


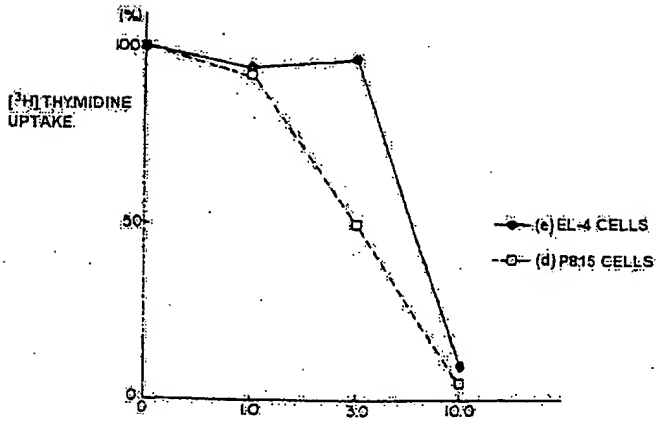
FIG. 1 (c)

GROWTH INHIBITORY ACTIVITY OF
GENISTEIN AGAINST SV40-3Y1 CELLS



DAYS OF CULTIVATION

FIG. 2



CONCENTRATION OF GENISTEIN (μg/ml)

Written Amendment (Voluntary)

May 23, 1985

Manabu Shiga, Esq.,
The Director General
of the Patent Office

1. Identification of the case:
   Patent Application No. 60(1985)-89770
2. Title of the invention:
   CARCINOSTATIC AGENT
3. Person making amendment
   Relationship to the case: Applicant for patent
   Address: 2-5-1, Nihonbashi Honcho, Chuo-ku, Tokyo 103
   Name: (667) Yamanouchi Pharmaceutical Co., Ltd.
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   Name: (9094) Seiya Fujino and one other
5. Item to be amended:
   Column of "Detailed Description of the Invention" in the specification
6. Subject matters of amendment:
   As per attached paper.

(1) In the specification on page 4, line 2, amend "(a) and (b)" to "(a) to (c)".

(2) In the specification on page 6, line 3, amend "Src" to "src".

(3) In the specification on page 7, line 2, amend "Src" to "src".

(4) In the specification on page 8, line 10, amend "reacted" to "was allowed to react".

(5) In the specification on page 9, line 7, amend "adjusted" to "prepared".

(6) In the specification on page 9, line 1 from the bottom, amend "adjusting" to "preparing".

(7) In the specification on page 10, line 8, amend "adjusted" to "prepared".

(8) In the specification on page 11, line 3, amend "adjusted" to "prepared".

(9) In the specification on page 11, line 10, amend "reacted" to "was allowed to react" and amend "mouth paper" to "filter paper".

(10) In the specification on page 11, line 11, "mouth paper" to "filter paper".

(11) In the specification on page 11, line 11, delete "with".

(12) In the specification on page 13, line 10, amend "adjusted" to "prepared".

(13) In the specification on page 14, line 11, amend "for 3 min" to "for 30 min".

evoked oto-acoustic emissions. *Arch Otolaryngol Head Neck Surg* 1988;114: 887-90.
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Catheterisation: your urethra in their hands

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Br Med J 1990;301:905

The emphasis in undergraduate medical education is often on the theoretical aspects of medicine rather than the practical aspects. Practical procedures are commonly taught informally, the teaching being passed from one junior to the next.[1] The philosophy is of "See one, do one, teach one." Urethral catheterisation is a procedure that requires a certain amount of skill, knowledge, and experience and is not without complication,[2-4] yet it is usually delegated to the most junior and inexperienced medical staff, the junior house officers.

Subjects, methods, and results

To assess the level of competence at catheterisation among junior medical staff house officers at this hospital were interviewed with a structured questionnaire, covering three aspects of the procedure: the degree of undergraduate and postgraduate instruction, the practical and theoretical aspects of catheterisation, and, finally, problems and complications encountered.

Thirty junior house officers (graduates of five medical schools) were interviewed. Eighteen were male and 12 were female. The replies to the questionnaire showed that none of the interviewees had received any formal instruction regarding any aspect of urethral catheterisation as an undergraduate. Practical postgraduate instruction in 24 was limited to supervision of a single catheterisation, and four subjects were unsupervised. Although those interviewed had performed a mean of 28 (range 6-100) catheterisations in male patients, only four of them had catheterised female patients.

Despite the large number of procedures performed there was appreciable ignorance of the practical and theoretical aspects of catheterisation. Twenty five interviewees were unaware of the availability of short term and long term catheters or of the duration for which they may be safely left without being changed. Three interviewees simply used the catheter that was provided by the nursing staff, and one did not know that different sizes existed.

Twenty eight interviewees initially used force when meeting resistance to the passage of the catheter, and 13 stated that the development of fresh urethral bleeding would not deter them from a further attempt at catheterisation. Eighteen were happy to attempt catheterisation in a patient who had a known urethral stricture. Five interviewees were unaware of the difference between a phimosis and paraphimosis.

Despite the lack of formal tuition all had developed what seemed to be a satisfactory aseptic technique. None, however, was aware of the nature of the antiseptic fluid or the strength of the local anaesthetic gel, but simply used what was provided by the nursing staff.

Nineteen of the interviewees had encountered bleeding and six had had patients in whom a paraphimosis had developed after catheterisation. A particularly disturbing finding was that, although 14 interviewees had requested help from senior medical staff, seven were reluctant to seek advice, because of their impression that difficulties with catheterisation were not worthy of disturbing senior staff. Eight of the 12 female medical staff had encountered problems with male patients becoming sexually excited during the procedure.

Discussion

The results of our survey suggest that the technique of urethral catheterisation is poorly taught, and in the light of these results we are preparing a short teaching video to be shown to every house officer at the start of their preregistration post.

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Oestrogenic effects of plant foods in postmenopausal women

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Crops grown as animal pasture are known to have oestrogenic activity,[1] and some foods contain potential oestrogenic analogues such as the isoflavonoids (isoflavones and coumestans), lignans, and resorcyclic acid lactones,[2] which may be activated or inactivated.[3] We studied the effect of three foods reported to induce vaginal oestrus in laboratory animals[4] in postmenopausal women not taking oestrogen replacement therapy.

Subjects, methods, and results

We studied 25 postmenopausal women who were non-smokers, in good general health, and taking no drugs known to affect oestrogen state (mean age 59 (range 51-70); body mass index 24·4 (range 18·7-31·6) kg/m^2; years after menopause 8·1 (range 1-20)). The protocol was a latin square design with a two week run in period and a six week experimental period. The women recorded their normal diet for 14 days and were asked to repeat the fortnightly diet throughout the study. During the experimental period the diet was

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supplemented with soya flour (45 g daily), red clover sprouts (10 g dry seed daily), and linseed (25 g daily), each for two weeks in turn. To check compliance the women returned residual food. Blood samples were taken weekly and lateral wall vaginal smears taken fortnightly and at follow up two and eight weeks after supplementation finished. Analysis was on intention to treat, but 23 women completed the study.

We examined the dependent variables vaginal cell maturation and serum concentrations of luteinising hormone and follicle stimulating hormone. The cumulative effects of the three foods at six weeks were compared with baseline by the paired *t* test, as were the residual effects, two and eight weeks after the last food supplement. We found significant differences in vaginal cytology after six weeks' supplementation ($p<0.01$, 95% confidence interval 6·0 to 17·6), which persisted for two weeks after treatment ($p<0.02$), but cytology returned to baseline after eight weeks (table).

Mean (SE) values for oestrogenic indicators in postmenopausal women consuming phyto-oestrogens

Week		Maturation value	Luteinising hormone (IU/l)	Follicle stimulating hormone (IU/l)
1			45·7 (3·1)	58·7 (2·9)
2		30·8 (4·5)	46·6 (3·4)	58·7 (3·0)
3			50·8 (8·5)	57·4 (2·9)
4		35·0 (5·1)	46·0 (3·6)	57·3 (2·9)
5	Food		46·2 (3·3)	57·7 (3·0)
6	supplementation	39·6 (5·3)	42·9 (3·2)	54·3 (2·9)
7			43·6 (3·3)	56·4 (2·8)
8		43·4 (3·6)	44·6 (3·3)	56·6 (2·4)
9			44·9 (3·5)	57·9 (2·8)
10		43·6 (4·7)	44·9 (3·3)	57·5 (2·7)
16		33·7 (5·5)		

The maturation value significantly increased after soya flour ($p<0.05$) and linseed ($p<0.02$) but not after red clover sprouts ($p=0.11$).

All women had concentrations of follicle stimulating hormone and luteinising hormone greater than those in the premenopausal range of 2-8 IU/l and 6-13 IU/l respectively. There was a cumulative effect on serum concentrations of follicle stimulating hormone ($p<0.05$) but not on luteinising hormone over the six week supplementation period. Individual two week food supplements had no measurable effects on either hormone.

In seven women with the most pronounced changes in vaginal cytology we measured serum oestradiol concentrations weekly. Baseline concentrations were <70 pmol/l in all but one woman, who was retained as the study was based on intention to treat. There were no appreciable changes in body weight during the study.

Comment

We aimed to consider whether phyto-oestrogens were of consequence in human nutrition. Our study gives some indication of the recovery time from any possible effect of treatment and also provides further evidence of causality. Vaginal maturation is a sensitive and specific indicator of oestrogenicity. Follicle stimulating hormone is less sensitive to weak oestrogenic compounds such as phyto-oestrogens. Weak oestrogenic compounds may sometimes act as anti-oestrogens, which may affect their usefulness as sources of oestrogenic activity. Conversely, tamoxifen, an antioestrogen, can have oestrogenic effects on vaginal cytology.[5]

Patterns of food intake may modulate the severity of the menopause as it is an oestrogen deficiency state. Up to half of the diet of some populations may comprise foods containing phyto-oestrogens, whereas in our study such foods comprised only about 10% of energy intake for a fairly short time. Whether menopausal symptoms differ in such populations would be worth investigation.

We thank our statistical adviser, Steve Farrish, from the department of social and preventive medicine, Monash University.

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Inadvertent duplicate publication

Loop diathermy excision of the cervical transformation zone in patients with abnormal cervical smears

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The *BMJ* regrets that much of the material in the above article (30 June 1990, p 1690) was substantially the same as that published previously in *Contemporary Reviews in Obstetrics and Gynaecology* (Redman CWE, Buxton EJ, Cullimore J, Luesley DM. Loop diathermy excision of the cervical transformation zone in the management of cervical intraepithelial neoplasia. 1990;2:53-8). The authors did not tell us this when the article was submitted, their article did not contain any reference to the earlier paper, and all authors signed our copyright form, which states, among other things, that "papers are accepted on condition that they have not been published by any other journal."

We regret this inadvertent duplicate publication, for which the authors hold sole responsibility, and which is in violation of our Instructions to Authors and internationally agreed guidelines.

Correction

Incidence of peptic ulcer disease in Gothenburg, 1985

An editorial error occurred in this paper by Dr Ivi-Mai Schöön and others (1989;299:1132). The y axis of figure 1 should read 0, 5, 10, 15, and 20 and not 0, 0·5, 1·0, 1·5, and 2·0 as published.

• *Reproductive Toxicology Review*

REPRODUCTIVE AND GENERAL METABOLIC EFFECTS OF PHYTOESTROGENS IN MAMMALS

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Key Words: Phytoestrogens, Mammalian reproduction, Reproductive hormones, Gonadal steroids, Luteinizing hormone, General Metabolism, Ovarian function, Reproductive neuroendocrinology.

INTRODUCTION

Historically, phytoestrogens were first investigated when it was noted that ewes that grazed Australian clover pastures for prolonged periods of time became sterile. It was found that the active agents in the clover that precipitated sterility were estrogenic (1). Later a similar phenomenon was observed to occur in the California quail during dry years, when phytoestrogen concentrations in available forage were increased (2).

Phytoestrogens are defined as plant substances that are structurally and functionally similar to the gonadal steroid 17β-estradiol (E_2) or that produce estrogenic effects (3). There are three main groups of nonsteroidal dietary estrogens. Phytoestrogens include the isoflavones (i.e., genistein, genistin, daidzein, biochanin A, formononetin, and pratensein) and the coumestans (i.e., coumestrol and 4'-o-methylcoumestrol). Mycoestrogens of the resorcylic acid lactone group (i.e., zearalenone and zearalenol) are also commonly found (4). The structural similarity between these substances, endogenous mammalian estrogens (E_2 and estrone), and potent synthetic estrogens (diethylstilbestrol) have been studied (Figure 1). Isoflavones, the monocarboxylic derivatives of the 15-C flavones, and coumestans contain central structures of 15 carbons. Both of these groups are derivatives of 3-phenylchroman (Figure 1) and thus may be considered a single family of compounds (5). The fungal resorcylic acid lactones and endogenous estrogens possess central structures of 17 carbons.

The similarity among these compounds has led investigators to study the possibility that phytoestrogens might act on physiological processes and behavioral patterns to alter reproductive performance (3). If reproductive effects occur, then these compounds might have a role in the evolutionary success of herbivores, perhaps making the difference between survival and extinction for some species. It is possible that phytoestrogens, through mimicry of endogenous animal estrogens, function as defensive substances by which plants diminish the fertility of herbivores which feed on the plants (6). In effect, the phytoestrogens may be seen as one of the many variables determining animal fitness for survival. This argument is supported by noting that animal species differ in their sensitivity to phytoestrogens (7). Some species are relatively resistant to the estrogenic effects of these compounds, while others may suffer sterility as a result of prolonged ingestion of phytoestrogens. We have hypothesized that phytoestrogen-induced physiologic and behavioral effects in mammals are significant factors in the reproductive and therefore evolutionary success of the consuming species. We have initiated our analysis of this broad hypothesis by reviewing the available data relevant to the reproductive and general metabolic effects of phytoestrogens in mammals.

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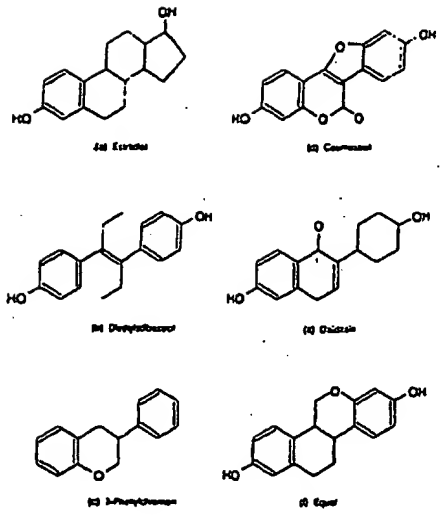

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PHYTOESTROGEN EXPOSURE

Sources of phytoestrogens

Phytoestrogens are produced by numerous Leguminosae and grasses, including many plants commonly consumed by man and livestock (Table 1). The estrogenic components are found in differing amounts in all parts of the plant, including the seeds, the flowers, the leaves, the roots, and the fruits. Concentrations in each tissue depend on plant type (4,8).

Of particular interest regarding possible human exposure is the presence of phytoestrogens in marijuana and coffee. It had long been suspected that the estrogenic effects of marijuana were due to Δ^9-tetrahydrocannabinol (THC), the major psychoactive compound. Smoking of marijuana significantly suppresses luteinizing hormone (LH) levels

Table 1. Some common plants that contain estrogenic substances

Alfalfa	Coffee	Oats	Rice
Anise	Date Palm	Orchard grass	Rye
Apple	Fennel	Palmetto grass	Sage
Barley	French Beans	Parsley	Sesame
Blue grass	Garlic	Peas	Soybean
Carrot	Green Beans	Pomegranate	Soya sprouts
Cherry	Hops	Potato	Wheat
Clovers	Liquorice	Rape	Yeast
	Marijuana	Red Beans	

during the human menstrual cycle and shortens both the menstrual cycle and the luteal phase (9). Since these results agree with observations in ovariectomized rhesus monkeys injected intramuscularly (i.m.) with THC, it was assumed that the menstrual cycle effects of smoke inhalation would be exclusively due to the THC content of the smoke (10). However, crude marijuana extract and condensed marijuana smoke compete with estradiol for estrogen receptors in the uterus of rats, while in vitro studies detected no binding of cannabinoids to estrogen receptors (11). These findings show that marijuana contains estrogenic substances that may be affecting reproductive processes via cannabinoid-independent mechanisms. Furthermore, apigenin, a derivative of flavonoid phytoestrogens found in crude marijuana, is a moderately potent inhibitor of estradiol binding to uterine estrogen receptors (11). Differentiation between the suppressive effect of THC on LH and the estrogenic effects of marijuana *per se* remains unclear.

Another plant product which is commonly ingested for pleasure rather than nutrition is coffee. Like marijuana, coffee contains weakly estrogenic constituents, evidenced by the estrogenic effects of increased uterine-to-body weight ratio and total uterine protein content following administration of coffee extracts by gavage (12). Ultraviolet absorbance spectroscopy suggests that whatever this active compound may be, it does not belong to one of the three major classes of dietary estrogens (e.g., flavonoids, coumestans, or resorcylic acid lactones). Thus, coffee may contain an estrogen precursor that requires metabolic activation or a structurally unrelated estrogenic compound.

Metabolism, distribution, and clearance

The relative potency of a phytoestrogen depends upon the target tissue, functional state of the target tissue, the animal species involved, and the route and pattern of delivery. In addition, the fami-

lies of estrogenic compounds that occur in plants can be modified by metabolism within the herbivore or even by gut flora prior to uptake. Dietary isoflavone phytoestrogens undergo bacterial modification in the gastrointestinal tracts of animals to yield equol, a weak, nonsteroidal phytoestrogen (8,13,14). Following ingestion of estrogenic plants, a temporary 50- to 1000-fold increase in urinary equol takes place, while insignificant traces of the initially consumed phytoestrogens appear in the urine. Noteworthy is that the major urinary product following the consumption of genistein and biochanin A is p-ethyl phenol, and formononetin consumption yields both daidzein and equol as the major urinary products (4). Furthermore, gut microflora (14) convert daidzein to equol which in turn is absorbed and enters the enterohepatic circulation. Notably, it appears that not all people have the ability to convert other isoflavones to equol. This may be due to the absence of bacteria capable of the conversion of precursors to equol (as is the case in the sterile gut of newborns), the composition (subpopulations) of intestinal microflora present, the intestinal transit time, pH, or redox potential. These factors may be influenced by diet, host immunity, medication use, etc.

Receptor activity and interaction with endogenous estrogens

Phytoestrogens exhibit binding to endogenous estrogen receptors. Binding of phytoestrogens to estrogen receptors is supported by the finding that the larger the dose of phytoestrogen given an organism, the greater the displacement of bound tritiated (^{3}H) E_2 (15). It has also been reported that at very high dosages, all phytoestrogens exhibit more than 80% competitive binding to renal tumor cytosolic estrogen receptors (16). The structural requisites for estrogen receptor binding are met by phytoestrogens. For example, equol possesses a potency on the order of 10^{-3} the estrogenic activity of E_2 and contains a phenyl substituent also present in E_2 and in DES (Figure 1). The substituent considered to be a requirement for estrogenic activity is a hydroxyl group in the same position as the hydroxyl group in the benzene ring of E_2 (14). Another structural similarity which facilitates estrogen receptor binding activity of equol and other phytoestrogens is that the distance between C-3 and C-17 in E_2 is about equal to that between the two hydroxyls in equol.

Considering the large quantities of phytoestrogens ingested by many mammals including man, functionally significant estrogen receptor occupancy by phytoestrogens occurs. Since no phytoestrogen has receptor affinity equal to that of E_2 and the degree of DNA stimulation due to phytoestrogens appears to be substantially less than that evoked by E_2 (8). phytoestrogen actions could be either estrogenic or anti-estrogenic. In a relatively hypoestrogenic individual, receptor occupancy by weak (exogenous) estrogens would likely produce estrogenic effects, while in a normally estrogenized individual, large amounts of weak estrogens might diminish the effective estrogenic activity by competition with E_2.

REPRODUCTIVE EFFECTS IN MAMMALS

Phytoestrogens have been shown to influence virtually every aspect of the mammalian reproductive process via effects on the morphology and physiology of reproductive organs and alteration of sexual behavior. The changes may be reversible or irreversible, depending on the duration and dose of exposure to the phytoestrogens.

Cervix

A pubertal pattern of cell differentiation has been noted in ewes rendered sterile by chronic ingestion of phytoestrogens (17). Among these changes, the cervix assumes a uterine pattern. Folds present in the cervix fuse, resulting in loss of cervical crypts, and the cells of the lamina propria become like those of the uterine stroma. Furthermore, glands having histochemical reactions reminiscent of uterine glands become plentiful in the cervix. Such an increase in abnormal glands may be responsible for the different composition which the cervical mucus takes in sheep with "clover disease." At low phytoestrogen dosage, the cervical mucus has a lower viscosity, not due to a higher water content; but rather due to a decreased concentration of glycoprotein — the component of mucus that affords its consistency. The level of glycoprotein seems to respond to the duration of exposure to the phytoestrogen rather to the dosage of the agent. This change in the cervical mucus compounds the anatomical compromise of the cervix such that the cervical reservoir for sperm in the ewes is greatly reduced. Since sperm recovered from the cervices of clover-affected ewes exhibit decreased motility (17), it appears that the phytoestrogen effect makes the mucus relatively "hostile" in the classic sense of cervical factor infertility. Such spermatoxicity is not understood in general nor in this specific case.

At higher phytoestrogen dosage, both higher volume and water content of cervical mucus are

observed in ewes (17,18), thus indicating that both cervical glycoprotein production and water excretion in the mucus are affected.

The cervical effects of phytoestrogens likely depend upon estrogen receptor mediation. In ewes, phytoestrogen treatment increases the rate of protein and glycoprotein synthesis and the number of estrogen binding sites in the cervix, but binding affinity remains unchanged (19). This finding implies that exogenous estrogen not only occupies the available binding sites, but stimulates the local production of more sites. Such receptor "up-regulation" may make the tissue more sensitive to estrogen action, and, if estrogen exposure continues, the cervical alterations would become more exaggerated.

Uterus

Pronounced uterine effects of phytoestrogens are also observed. The most notable uterine change that occurs is a marked increase in its weight relative to body weight, which constitutes the classic bioassay for estrogen action. A dose-dependent uterine weight increase is precipitated by acute administration of an extract of the Indian herb *Achyranthes aspera* in rats and hamsters at contraceptive dosage (75 mg/kg) and with as little as 1/20 this dosage (20). Similar results have been observed in mice, rats, and hamsters with only 1/40 contraceptive dose of ferujol extract (21). Stob (4) suggests that this hypertrophy of the uterus is the result of "typical estrogenic mechanisms," implying estrogen-receptor mediation. However, a more complex response to daily s.c. injection of female lambs with the phytoestrogen β-sitosterol has been reported, in which uterine weight increases for the first two weeks of treatment but markedly decreases over the next six-week period (22). Plausible explanations for such biphasic results include receptor "down regulation" and induction of metabolic enzymes with enhanced clearance of β-sitosterol. Similar results were obtained using ovariectomized ewes as the model (23).

Another manifestation of the uterotropic effect of phytoestrogens is seen in ewes suffering from infertility due to prolonged exposure to these agents. A marked increase in activity of some uterine enzymes and uterine DNA, protein, and glycoprotein synthesis occurs in such sheep (19). This observation indicates that at least a portion of the uterine weight gain is true hypertrophy rather than simply edema. At the same time, lower levels of lipids within the uteri of sheep fed phytoestrogen suggest inhibition of synthesis or increased utilization of lipids within this organ (22). Thus phytoestrogens may be affecting different enzymes in different fashions, stimulating the activity of some while blocking the action of others. It is noteworthy that the uterine RNA-to-DNA ratio decrease that occurs following ovariectomy is smaller in clover-affected than in normal ewes. This response is accompanied by less regression of the uterus in clover affected ewes than in controls. These findings indicate that phytoestrogenic action may be mediated via differentiations similar to those induced by hormonal steroids during fetal development (24).

Gross structural lesions of the uterus may also result from phytoestrogen exposure and could account for some instances of permanent sterility. Lesser lesions entail the proliferation of cystic endometrium, myometrial fibrosis, and endometrial fibrosis (13). These lesions could certainly compromise normal implantation of the conceptus. The most severe structural failure, complete uterine prolapse, is known to occur in some species following ingestion of some dietary estrogens (mycoestrogens) and obviously disrupts the reproductive process.

It is not clear whether phytoestrogens play any role in pregnancy wastage, but some plant preparations have been used as abortifacients. *Achyranthes aspera*, a common Indian herb claimed to possess abortifacient activity, did induce abortion in mice and rabbits, but failed to show similar effects in rats (20). It is uncertain whether a phytoestrogen is the active agent of *Achyranthes* that brings about abortion, but support for that possibility derives from the finding that miroestrol, a phytoestrogen from a legume tree root, is used by Burmese and Thai women in plant extract form to induce abortion (25). The mechanism for such an abortifacient action of these compounds is unstudied and any effects of phytoestrogens on uterine contractility *per se* have not been determined in either the gravid or nongravid state.

Phytoestrogen effects on uterine function may relate to alterations in activity of several enzymes. Under normal circumstances, oxidative enzymes in the uterus show slight reactions in the endometrium and uterine glands, but after administration of β-sitosterol, these weak reactions are curtailed (22). Such an inhibition of oxidative enzymatic activity in the uterine endometrium and glands may reduce local energy production due to an inability to replenish NAD+ and NADP+. This circumstance would diminish the ability of the uterus to contract and might decrease secretory capabilities of the uterine glands.

Alkaline phosphatase in the uterine tissue of ewes also responds to β-sitosterol in a biphasic pat-

term. Alkaline phosphatase activity increases over the first two weeks of daily β-sitosterol injections and decreases over the second two weeks of injections (22). This disturbance in alkaline phosphatase activity may alter cell permeability and transport of nutrients by uterine cells.

Acid phosphatase activity in the uterus decreases with increasing dose and time of daily β-sitosterol treatments over an eight-week span (22). Such an inhibition would decrease free phosphorous, and may relate to the more general observation of decreased plasma phosphorus levels in exposed animals.

Uterine cholinesterase activity also decreases following β-sitosterol treatment, as evidenced by its diminished activity towards acetylthiocholine (22). This inhibition of activity is accompanied by a downward shift in sodium ion transport and decreased sodium in the uterine luminal fluid. It is not clear whether effects on sodium transport and cholinesterase activity are coincidental or truly associated processes in this instance.

Ovaries

While many anatomical effects of phytoestrogens have been described, physiologic changes in the reproductive tract are more subtle, but perhaps more consequential. Ovarian cyclicity may be disrupted by phytoestrogen exposure in mammals and birds (2,14,25,26), but interruption of ovulation due to short-term phytoestrogen ingestion is reversible (26). It is plausible that human vegetarians may have ovulatory dysfunction but suffer no other obvious physiologic abnormalities due to their diets (14). Abnormalities of ovulation may be due to direct ovarian actions since administration of β-sitosterol to ewes inhibited follicular development and altered the size distribution of follicles (22). Follicles were observed to show degeneration with intrafollicular hemorrhage and the development of shrivelled oocytes with lipid inclusions. The suggestion of a direct ovarian action of phytoestrogens in perturbing follicular maturation may be supported to some extent by a study which showed that in rats intraperitoneal administration of an extract from a plant species known to contain high concentrations of phytoestrogens inhibited follicular maturation (26). Obviously, these studies cannot distinguish between direct ovarian and indirect effects on follicular growth.

More direct evidence that the follicle may be a site of phytoestrogen activity derives from in vitro cultures of bovine granulosa cells. In this system, lower dosages of genistein and biochanin A increased progesterone synthesis while higher dosages inhibited progesterone synthesis (27). Since progesterone is essential in the establishment and maintenance of pregnancy, such an inhibition of progesterone production would be a plausible explanation for both failure of conception and early pregnancy wastage.

The possibility that phytoestrogens might be toxic to oocytes or early embryos was suggested in a single study (7). Mice fed coumestrol and then mated produced degenerate embryos exhibiting unevenly distributed cytoplasm and lack of symmetry in size among blastomeres, suggesting alterations in cleavage rates. Extensive vacuolization found in the ova also suggests that failure of fertilization of these ova may account for part of the observed decrease in litter size in mice fed coumestrol.

The activities of two ovarian enzymes appear to be influenced by phytoestrogens. First, low doses of phytoestrogen inhibit 17,20-lyase in bovine granulosa cells (27). This effect could profoundly alter the pattern and capacity of the steroidogenic pathways within the follicle or corpus luteum. The precise mechanism by which this effect occurs is unproven. Second, alkaline phosphatase in the ovaries is affected by phytoestrogen exposure (22). While the overall alkaline phosphatase activity is about equal in the ovaries of β-sitosterol-treated and control ewes, the control ewes show an intense reaction in the zona pellucida with a weak reaction in the interstitial tissue. Treated ewes exhibit an opposite response. Thus, a reversal of activities is seen where phytoestrogen is acting both to stimulate and to inhibit the same enzyme in two different sites within the ovary. While a mechanism for this action is not known, such changes in the activities of ovarian enzymes might compromise ovulation and increase the incidence of follicular degeneration in animals treated with phytoestrogens.

CNS/pituitary

Some phytoestrogen effects on ovarian function appear to result from indirect action on the secretion of gonadotropic hormones (7). In this context, there are four possible mechanisms of phytoestrogen action: 1) they are E_2 agonists, 2) they are E_2 antagonists, 3) they act as both E_2 agonists and antagonists, and 4) they act in a nonestrogenic capacity. Available information best supports the third of these possibilities (mixed agonist-antagonist effects). The site of phytoestrogen action could be the CNS (especially hypothalamus), the pituitary, or the gonad (see previous section).

The effect of intraperitoneal injection of phytoestrogen-rich *Dieffenbachia amoena* extract in rats on LH, follicle-stimulating hormone (FSH), prolactin (PRL), progesterone, and E_2 have been studied (26). In treated rats, levels of LH, FSH, and progesterone increased for doses of 2.5, 5.0, and 10.0 mg/kg of extract, while the levels of PRL and E_2 decreased at the same dosages. Progesterone levels showed a biphasic response, increasing at low doses of the extract (26), but decreasing at higher doses (27). Since no obvious single mechanism would explain all of these pituitary and ovarian hormonal changes, the extract may contain more than one endocrinologically active substance, or more than one site or mechanism of action might be involved.

There are data to suggest that phytoestrogens act both at CNS and pituitary levels to alter gonadotropin secretion. In both ovariectomized ewes (23) and intact clover-affected ewes (17), the best explanation for the impairment of gonadotropin secretion was a hypothalamic/CNS action. In particular, in clover-affected ewes, an LH surge could not be elicited by exogenous E_2 administration (consistent with loss of positive feedback), but the LH secretory response to exogenous gonadotropin-releasing hormone was normal (17), suggesting no pituitary effect. Our own data (28) show that acute phytoestrogen administration can alter GnRH-induced LH secretion in ovariectomized rats and thus suggest that the pituitary may be a site of phytoestrogen action in other situations.

Interactions between reproductive effects of phytoestrogen exposure and photoperiod in seasonal breeders have been investigated. In normal intact ewes, the frequency of LH pulses and plasma LH concentration are higher during breeding season than during anestrus season. In clover-diseased ewes, the frequency of LH pulses and LH concentration during breeding season are nearly the same as in normal ewes. In contrast during anestrus season, these LH pulse parameters remain at the high level of breeding season in clover-affected ewes, rather than decreasing as in normal ewes (18). These results suggest that a dissociation of normal photoperiod controls from the LH pulse generator may result from prolonged phytoestrogen exposure.

In ovariectomized ewes given estradiol implants, LH pulse frequency and amplitude vary seasonally, rather like the pattern seen in intact ewes. This seasonal variation in LH pulse frequency in ovariectomized ewes could depend upon extraovarian steroids from the adrenal glands, other intrinsic photoperiod-dependent CNS functional changes, or dietary estrogens. Results from one study suggest that dietary coumestrol decreases the amplitude of LH pulses but fails to affect the frequency of LH pulses or FSH concentrations during the breeding season (23). During anestrus, coumestrol does not alter any of these variables. Thus, coumestrol could only be partially responsible for the seasonal decrease in LH pulse frequency in ewes.

Sexual behavior

Changes in sexual behavior due to phytoestrogen exposure parallel the known physiologic effects. Clover-diseased ewes are slower than normal ewes to exhibit estrus behavior in response to either a single or several daily doses of E_2 (17,29,30). Accompanying the delayed estrus is a retarding of the first mount of the ewes by the ram, although the number of days on which the ewes allowed the ram to mount them does not significantly differ from controls. A delay of estrus in mice fed coumestrol also occurs (7), implying an antiestrogenic effect.

Apparent defeminization of the sexual behavior response following consumption of phytoestrogens is displayed by clover-affected ewes. These ewes show aggressive behavior, such as challenging and head bunting of rams and other ewes, sooner than control ewes following administration of testosterone (17). At the same time the ewes are slower in showing female behavior, such as standing to be mounted by a ram. Furthermore, clover-affected ewes exhibited less soliciting behavior than normals. However, the number of ewes that stood to be mounted decreased equally over the five-week period during which daily testosterone injections were given (30). Relative to controls, clover-diseased ewes exhibit a significantly greater degree of courting behavior 28 but not 21 days following treatment with testosterone. Other courting behaviors that are less hormonally dependent, such as anal and genital sniffing by the ewes, are not altered (17,30). While mechanisms for these behavioral effects are not known, we do know that females and males have similar numbers of estrogen binding sites in the hypothalamus, but estrogen-receptor complexes appear to have shorter nuclear acceptor occupancy in males than in females (31). Behavioral changes in clover-affected ewes could result from a change as simple as a decrease in nuclear acceptor occupancy by estrogen-receptor complexes.

E_2 causes a dose-dependent increase in the incidence and duration of hormone-dependent behaviors in ewes (Table 2), whereas E_2 has no effect on hormone-independent behaviors (30). The E_2

Table 2. Estradiol-dependent and -independent behaviors in ewes

Hormone-dependent behaviors	Hormone-independent behaviors
Active soliciting	Squatting
Standing for mounting	Looking over shoulder
Allowing ram to mount	Tail fanning
	Kicking

induced behaviors occur less in phytoestrogen-affected ewes than in normals, while E_2 independent behaviors occur with equal frequency in control and clover-diseased ewes. Since general behavior appears normal but female sex-specific behavior is compromised in phytoestrogen-treated ewes, reproductive success could be compromised on a behavioral basis. The relationship of phytoestrogen-induced anatomic changes in the external genitalia and sexual behavior is not defined, but coital biomechanics could be altered as a result of such end organ effects. While vulvar and vaginal hypertrophy has been noted in various animals, masculinization has been observed in ewes (17) with clitoromegaly and fusion of the ventral commissure. Upon removal from estrogenic pasture, these changes do not reverse and could, therefore, permanently alter sexual function.

Phytoestrogenic effects in males appear to be consistent with expectations for exogenous administration of bioactive estrogen. Coumestrol increases teat length in wethers (23) and stimulates mammary hypertrophy in intact males. Rams grazed on estrogenic clover have reduced sperm counts (14), but it is not clear whether fertility is affected.

GENERAL METABOLIC EFFECTS IN MAMMALS

Protein synthesis

Some data suggest that phytoestrogens affect levels of plasma proteins. The effects of β-sitosterol on plasma concentrations of albumin, alpha-globulin, beta-globulin, gamma-globulin, and fibrinogen have been studied (32). Normal functions of these proteins are indicated in Table 3 (33). Even though total plasma protein concentration in mice is unaffected by s.c. administration of β-sitosterol, daily 25 to 100 μg injections of the agent increase four of the plasma proteins, but significantly decrease the gamma-globulin complex. The mechanisms of action of phytoestrogens in this system

Table 3. Plasma protein fractions affected by β-sitosterol*

Protein	Function	Effect of β-Sitosterol
Serum albumin	Regulation of blood volume; transport of fatty acids	Increase
Alpha-globulins	Transport of lipids, thyroxine, adrenal cortical hormones, and copper	Increase
Beta-globulins	Transport of lipids, iron, and hemes	Increase
Gamma-globulins	Act as most of the circulating antibodies	Decrease
Fibrinogen	Precursor to fibrin of blood clots	Increase

*(See reference 32).

are not established. It is likely that the phytoestrogens stimulate hepatic protein synthesis but inhibit production of gamma-globulins by lymphoid tissues. It is possible that the increased alpha-globulin concentration is a compensatory occurrence to erythrocyte count reduction that occurs following administration of β-sitosterol, thereby maintaining normal blood viscosity in the absence of normal erythrocyte concentration. The increase in the beta-globulin–fibrinogen complex appears to be correlated with its affinity for binding phosphorus. This affinity increases in response to β-sitosterol (32).

Enzyme activity of the liver

Phytoestrogens influence enzymes in nonreproductive as well as reproductive tissues. A relation between diet and synthesis of three enzymes in the liver of cheetahs has been shown. The affected enzymes, alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyltransferase, decrease in amount when cheetahs are taken off a diet high in soya bean content (thus high in phytoestrogen content) and given a chicken diet (13).

Inorganic plasma constituents

Phytoestrogens induce mineral changes in the blood. Subcutaneous injections of 25, 50, 75, or 100 μg of β-sitosterol increase calcium levels in mice, while doses of 5 or 10 μg of the phytoestrogen have no effect on calcium levels (34). Since E_2 inhibits bone mobilization, β-sitosterol may act by causing a decrease in E_2 levels via inhibition of gonadotropin secretion from the pituitary. Decreased ovarian E_2

production might then result in increased bone mobilization and increased serum calcium. Surprisingly, blood plasma phosphorus levels decrease following administration of 5 to 75 μg doses of β-sitosterol in mice, but show little change in response to a 100 μg dose (34). Decreases in phosphorus could be due to an enhanced rate of storage in an extravascular compartment, increased utilization of phosphorus by tissues, or increased renal clearance.

While β-sitosterol doses of less than 5 μg fail to change plasma magnesium levels, higher doses decrease plasma magnesium and increase both hepatic and intramuscular magnesium (34). Since magnesium is a smooth muscle relaxant, changes in uterine or tubal smooth muscle motility could result indirectly from this phytoestrogen action.

PHYTOESTROGENS IN HUMAN DISEASE

Deleterious roles

Phytoestrogens have been suggested to play both deleterious and beneficial roles with regard to illness. In the diets of cheetahs, phytoestrogens cause vascular hepatic lesions, in which the centrilobular and sublobular hepatic veins are partially or totally occluded (13). The possibility of human hepatic dysfunction must therefore at least be considered.

Vascular disease may be correlated with the consumption of dietary phytoestrogens (35). Coronary heart disease has been suggested to be associated with phytoestrogens consumed indirectly through the milk of cows; that is, the lactating cow consumes the phytoestrogens while grazing and, in turn, phytoestrogens in cow's milk are consumed by humans. One basis for this proposal is that phytoestrogens have more structural similarity to DES, a potent synthetic estrogen found to have atherogenic properties, than to endogenous estrogens such as E_2. The higher rate of coronary heart disease in human males might be explicable in part if human females are found to be better able to metabolize and excrete phytoestrogens.

Dietary estrogens could be a factor in cancer initiation in hormone responsive tissues, but no such instances have been demonstrated. Certainly phytoestrogens bind to both rat and human mammary tumor tissue and show competitive binding for mammary tissue E_2 receptors (15) raising the possibility of stimulation of estrogen-dependent neoplasms.

Beneficial roles

Estrogens have two opposing effects on cancer, depending on dosage. Large doses inhibit breast cancer tumor development and suppress growth of tumors already present, but small doses seem to promote tumor development and stimulate growth (36). This duality extends to phytoestrogens. Phytoestrogens may stimulate or inhibit tumor growth (8,14). One mechanism by which phytoestrogens may manifest their antitumor effects is blockade of estrogen receptors and uncoupling of receptor-mediated response. Thus the ability of endogenous estrogens to support tumor growth would be reduced. Indirect demographic support for a phytoestrogen-mediated reduction in cancers of hormone-responsive tissues might derive from the observation that women in countries consuming vegetarian diets have a lower incidence of breast cancer than in societies where a meat and vegetable diet is consumed (37).

Phytoestrogens may have antiviral and fungicidal properties (37), but a mechanism is not known. Support for the notion that this group of compounds could have such properties may lie in noting that the antifungal drug, ketaconazole, is also a potent inhibitor of some steroidal enzymes.

Plant estrogens have been implicated in the reduction of serum cholesterol levels in humans and animals with hypercholesterolemia. Such action is likely related to the role estrogens play in the metabolism and interaction of lipoproteins with regulation of cholesterol (8).

A final beneficial phytoestrogenic effect is alleviation of vasomotor symptoms in menopausal women. Historically the Chinese have used herbal medicine to treat "hot flushes." These herbal medications work as well as Premarin (an equine conjugated estrogen) in the mitigation of these symptoms in women with natural menopause (38). Similarly, the mycoestrogen, zearalanol, has been reported to reduce the incidence of hot flushes in women with surgical menopause (4). These effects would be consistent with the expected estrogenic properties of these compounds.

CONCLUSION

Phytoestrogens influence mammalian reproductive processes and can thereby compromise the reproductive success of individual mammals and possibly function as a selective environmental factor for populations. While phytoestrogens have a few propitious effects, the majority of the effects are nocuous. These compounds act through their similarity to endogenous estrogens and compete with the endogenous estrogens for binding sites.

Short-term effects of phytoestrogens seem to result from their mixed agonist–antagonist effects on estrogen-mediated processes in mammals. Since long-term exposures can produce persistent, even permanent anatomic, physiologic, or behavioral changes, phytoestrogens must affect the differentiation of some reproductive tissues and irreversibly alter the integration of mammalian reproductive processes in susceptable species.

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Naturally occurring oestrogens in foods—A review

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This review is concerned with the presence of naturally occurring oestrogens in food plants and processed foods. Particular emphasis is placed on isoflavones and coumestans, both of which are true plant oestrogens, and the resorcylic acid lactones, more correctly classified as fungal oestrogens. The metabolism and mode of action of these compounds is discussed and their biological potencies, determined in both *in vivo* and *in vitro* studies, described. Current methods of analysis are indicated and the levels of these oestrogens in food plants, processed foods and feedingstuffs are presented. Botanical, environmental or technological factors affecting the possible intake of plant and fungal oestrogens are mentioned and the hazard associated with such intake is compared with that originating from other dietary or medicinal hormonally active substances. Indications are given of the wide range of common food plants which have been reported to possess oestrogenic (uterotropic) activity, although it is emphasized that in general further work is necessary to substantiate these claims and to confirm the identities of the biologically active principles which have in some cases been proposed. In the concluding section suggestions are made for additional research considered important or necessary in this interesting area.

Introduction

The presence in plants of oestrogens, compounds which induce oestrus in immature animals or interfere with normal reproductive processes, has been known for over half a century. However, the use of plants and plant extracts to control fertility in animals and humans has been recognized since earliest times. In the Orient, for example, the pomegranate has traditional associations with fertility which stretch back over 2000 years. Although many of the plant oestrogens have now been separated, purified and characterized, only occasionally have they been found to be identical with those of animal origin, oestrone (I) and 17β-oestradiol (II) (Hewitt *et al.* 1980) (see figure 1).

In 1954, Bradbury and White listed 53 plants which possessed the capacity to initiate oestrus in animals, but progress in this area was such that only two decades later Farnsworth *et al.* (1975) were able to describe over 300 such plants. In many cases the exact nature of the active principles has not been established but, of the identified compounds, isoflavones and coumestans are the most common. In all, these authors listed 29 plant oestrogens, many of which possessed structural similarity to synthetic diethylstilboestrol (III) (figure 1). Less than half the compounds listed have been reported in plants which are regularly consumed by animals or man. Such plants are listed in table 1 and it may be noted that certain of these, for example legumes and fodder crops, may be consumed in relatively large amounts. Indeed, problems of infertility in livestock (especially sheep) resulting from the grazing of oestrogen-rich pasture or fodders are a serious economic problem in many parts of the world (Hanson *et al.* 1965, Bickoff 1968, Shutt 1976) and have provided the stimulus for much of the

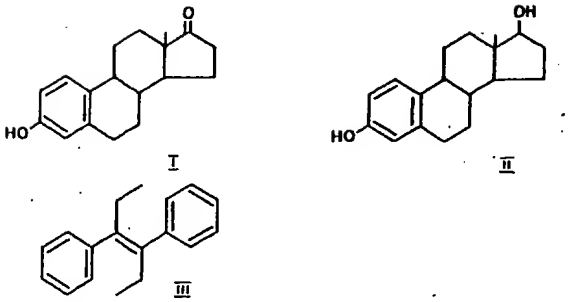


Figure 1. Structures of animal and synthetic oestrogens.

Table 1. Oestrogenic principles of edible plants.

Plant	Common name	Part	Active principle
Avena sativum	oats	seed, meal, sprouts	zearalenone[a] zearalenol[a]
Cicer arietinum	chick pea	seed, seedling	isoflavones
	Bengal gram		isoflavones
Daucus carota var. *sativa*	carrot		3-methyl-6-methoxy-8 -hydroxy,3,4-dihydro isocoumarin[b]
Foeniculum vulgare	fennel	oil	anethole[b]
Glycyrrhiza glabrata	liquorice	root	oestriol, β-sitosterol[b]
Hordeum vulgare	barley	embryo	zearalenone[a]
Humulus lupulus	hops		colupulon[b] lupulon[b] adlupulon[b]
Malus sylvestris	apple	fruit	oestrone
Medicago hispida	toothed medic		isoflavones
Medicago litteralis	barrel medic		coumestrol
Medicago sativa	alfalfa		4-methoxycoumestrol
Oryza sativa	rice	seed, embryo	zearalenone[a] oestrone, oestradiol
Phaseolus vulgaris	French bean	seedling	oestradiol
Phoenix dactylifera	date palm	seed	oestrone
Pimpinella anisum	anise	oil	anethole[b]
Poa pratensis	bluegrass		isoflavones
Prunus avium	cherry	fruit	prunetin
Punica granatum	pomegranate	seed	oestrone
Secale cereale	rye		zearalenone[a]
Sesamum indicum	sesame	meal	zearalenone[a]
Soja max	soya	seed	isoflavones
		sprouts	coumestrol
Sorghum vulgare	sorghum		zearalenone[a]
Triticum vulgare	wheat	flour, seed, germ oil	zearalenone[a]
Trifolium spp.	clovers	leaves	coumestrol
		stems	isoflavones
Vigna sinensis	cowpea		coumestrol
Zea mays	corn		zearalenone[a] zearalenol[a]
	hay		zearalenone[a]

[a] It should be emphasized that zearalenone and zearalenol are not produced by the plant *per se* but may occur on the plant as a result of synthesis by *Fusaria*.

[b] Tentative.

work which has been conducted on plant oestrogens. However, with the exception of those topics of relevance to the presence and effects of plant oestrogens in the human diet, e.g. studies on the analysis and metabolism of oestrogens and of their possible carry-over into the human body via the ingestion of animal products, the role of such compounds in fodders and other animal feedingstuffs will not be considered here.

Additional interest in naturally occurring oestrogens has resulted from the disquiet of scientists, consumers and legislators over the presence in meat and meat products of compounds, such as diethylstilboestrol, designed to improve animal growth and performance (Umberger 1975, McMartin *et al.* 1978). Although the biological activities of such compounds, expressed on a unit weight basis, are very much greater than those of plant oestrogens (see below), under normally regulated conditions their intake into the human body will be very much less. Since any health risk due to dietary factors is a consequence of both biological potency and exposure, there has in recent years been considerable study of plant oestrogens, their metabolism, modes of action and potencies. Such studies have revealed the considerable extent to which genetic, botanical and environmental factors determine the contents of these compounds and also how the processing of the raw plant prior to its consumption can exert similar effects. These studies have, in no small part, benefitted from the development of improved methods of chemical analysis, possessing greatly improved sensitivity and specificity. This paper reviews the more recent advances in these areas and identifies others awaiting additional investigation.

The major plant oestrogens

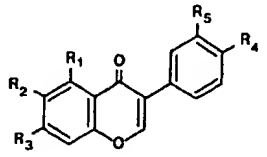
In this review, for convenience, the term 'plant oestrogen' will be used to describe all of the compounds considered in this section, although the resorcylic acid lactones have been referred to elsewhere as *fungal* oestrogens.

The biological effects of plant oestrogens (in the form of the purified compounds or as fresh plants, extracts or processed material) are generally demonstrated by measuring the uterine enlargement of immature female mice or the degree of cornification of the vaginal epithelium. Whereas the former is the more sensitive it lacks the specificity of the latter (Stob 1983); both assays are, however, subject to criticism and misleading results are possible (Emmens 1969).

Examination of table 1 reveals that compounds responsible for the oestrogenic activity mainly fall into three groups, according to their chemical structure. These are (*a*) isoflavones, which in many cases are present in the bound, glycosidic form; (*b*) coumestans; and (*c*) resorcylic acid lactones. A distinction can readily be made between the first two groups and the latter; isoflavones and coumestans are intrinsic plant components, although their levels are dependent upon many factors, including those associated with growth and genetic background. In addition, their levels may also be increased as a direct response to microbial or insect damage. In contrast, the resorcylic acid lactones are products not of the plant *per se*, but of *Fusarium* moulds which are common in the field and flourish in the warm, moist conditions of badly stored grains and other produce. Although other individual compounds possessing oestrogenic activity do occur in food plants, and are considered in the penultimate section of this paper, the major part is concerned with the above groups which are considered separately below.

Isoflavones and isoflavone glucosides

The naturally occurring isoflavones which have been shown to possess oestrogenic activity are (figure 2): daidzein (IV) and genistein (V), their glucosides, daidzin (VI) and



R_1	R_2	R_3	R_4	R_5	
H	H	OH	OH	H	IV
OH	H	OH	OH	H	V
H	H	O-glu	OH	H	VI
OH	H	O-glu	OH	H	VII
H	H	OH	OCH_3	H	VIII
OH	H	OH	OCH_3	H	IX
OH	H	OH	OCH_3	OH	X
OH	H	OCH_3	OH	H	XI
H	H	O-6′acetylglu	OH	H	XII
OH	H	O-6′acetylglu	OH	H	XIII
H	OCH_3	OH	OH	H	XIV
H	OCH_3	O-glu	OH	H	XV

Figure 2. Structures of naturally occurring isoflavones.

genistin (VII), and their 4′-methyl ethers, formononetin (VIII) and biochanin A (IX), respectively; two other active isoflavones, pratensein (X) and prunetin (XI) are of rather limited occurrence. It is possible that other derivatives may also possess uterotropic activity; for example, Japanese workers have isolated the 6′-O-acetyl derivatives of both daidzin and genistin (XII and XIII, respectively) from soyabeans, but they do not appear to have been assayed for their oestrogenic effects (Ohta *et al.* 1979, 1980). It is, however, likely that they are metabolized *in vivo* by ruminants and other animals to daidzin and genistin or their aglycones. Most of the above isoflavones occur in the intact plant in the bound form, as glucosides, but are readily degraded to the aglycone chemically or enzymically during processing, isolation and analysis. Bound isoflavones in clover and related pastures are readily hydrolysed by endogenous glycosidases when the intact plant is crushed (Beck 1964) and such hydrolysis can also occur in animals, and presumably man, in the absence of the plant enzyme. A large number of isoflavones have been isolated from plant species, but only a small number have been shown to possess oestrogenic activity. Moreover, not all isoflavones isolated from plants known to affect oestrus are active; for example, whilst soyabeans possess daidzein, genistein and their glycosides, they may also contain the uterotropically inactive glycitein (XIV) and glycitein-7β-glucoside (XV) (figure 2) (Naim *et al.* 1973).

The biological potencies of the individual isoflavones vary, but all are much less active than animal or synthetic oestrogens. Thus, although genistein is the most potent isoflavone in terms of its effect on mouse uterus (figure 3), it exhibits only 10^{-5} of the activity of diethylstilboestrol. The relative activities of the individual isoflavones vary with both the species and strain of animal used and with the route of administration. In sheep, biochanin A and genistein were about 20 times less active when introduced

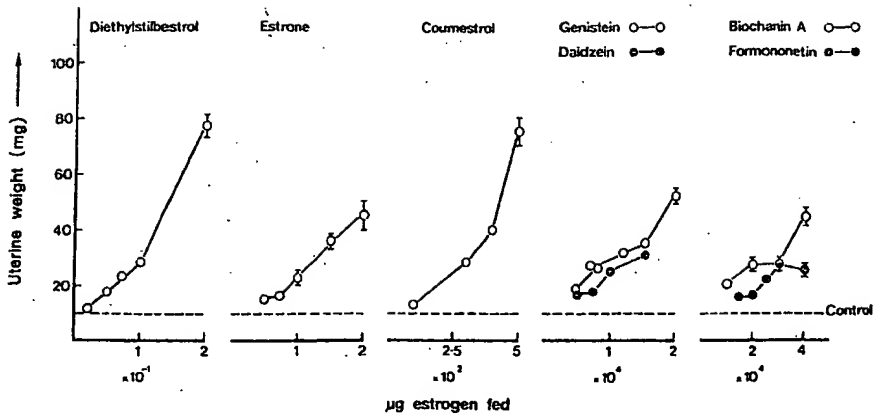


Figure 3. Relative uterotropic potency of diethylstilboestrol, oestrone, coumestrol and isoflavone oestrogens (after Stob 1983).

intraruminally as compared to intramuscular injection, whereas the latter route showed formononetin to be inactive (Braden *et al.* 1976). Genistein was found to be the most active of the isoflavone aglucones tested by oral administration in the mouse (Bickoff *et al.* 1962) and together with its glucoside was equally active when administered subcutaneously (Cheng *et al.* 1955). Differences in responses to other oestrogens between strains of mouse have, however, been reported (Fredericks *et al.* 1981) and recently Farmakalidis and Murphy (1984b) have shown the CD-1 mouse strain to be relatively insensitive to daidzin, genistin and genistein. Comparisons between data arrived at using different strains of mouse are, as the authors point out, thus to be treated with caution. Moreover, there would seem an obvious need to specify, and indeed standardize, the strain of mouse used in the uterotropic assay. Bickoff *et al.* (1962) have demonstrated that dietary isoflavones (daidzein, genistein) possessing a free 4′-hydroxyl group were more uterotropically active in the mouse than their 4′-methyl ethers (formononetin and biochanin A, respectively). The greater potency of genistein compared with daidzein has been attributed to interaction between the OH group and the adjacent carbonyl group of the latter (Bradbury and White 1954). The effect of pratensein is not included in figure 3, but it has been considered to be lower even than formononetin (Wong 1963).

Isoflavones, like the other main groups of plant oestrogens, exhibit an affinity for oestrogen receptor sites (Shutt and Cox 1972, Shutt 1976) and may therefore be considered to function as anti-oestrogens (Martin *et al.* 1978, Verdeal *et al.* 1980). (Anti-oestrogens are thought to exert their effect by decreasing the concentration of cytoplasmic oestrogen receptor and by complexing with the receptor, thus preventing biosynthetic processes associated with tissue development.) The affinities for the binding of genistein to rat, rabbit and sheep uterine cytosol are 1·3, 0·6 and 0·9 respectively (relative to 17β-oestradiol = 100). Other isoflavones are even less active: daidzein exhibits a relative binding affinity of 0·1 and 0·09 for sheep and rat uterine cytosol respectively; biochanin A has an affinity of 0·07 for rat uterine cytosol; and formononetin 0·01 for binding to sheep uterine cytosol (Verdeal and Ryan 1979). The isoflavone metabolites equol, O-desmethylangolensin and angolensin (see below) had relative affinities for sheep uterine cytosol of 0·4, 0·05 and 0·03 respectively (Shutt and Cox 1972).

Based upon the competitive binding to oestrogen receptors in steroid-binding globulins from human breast cancer cells (line MCF-T) the affinities of genistein and formononetin, relative to 17β-oestradiol, are 2 and 0·01 respectively (Martin *et al.* 1978). It seems most likely, as Verdeal and Ryan (1979) have suggested, that transport and metabolic effects are responsible for the apparent discrepancy between the results of the above affinity bioassays and those based upon uterotropic activity. The effects of pure isoflavones in the mouse, rat and sheep are summarized in table 2.

Table 2. Effects of pure isoflavones.[a]

Animal	Compound	Dose	Effect
Mouse	biochanin A	10–40 mg/g diet	uterine hypertrophy
	daidzein	5–15 mg/g diet	uterine hypertrophy
	formononetin	15–40 mg/g diet	uterine hypertrophy
	genistein	5–20 mg/g diet	uterine hypertrophy
	genistein	15 mg/day, diet	infertility, both sexes
	genistein	10 mg injected	displacement of oestradiol from uterine receptors
	genistin	5 mg/day, diet	uterine hypertrophy
	genistin	0·2% diet	infertility, females
	genistin	9–72 mg/day, diet	testes atrophy, depressed growth
Rat	genistein	0·5% diet	testes atrophy, depressed growth
	genistein	0·4 mg, injected	increased protein, phospholipid synthesis in uterus
	genistin	0·5% of diet	testes atrophy, depressed growth
Sheep	biochanin A	1 g, injected	uterine hypertrophy
	formononetin	24 g, injected	uterine hypertrophy
	genestein	1 g, injected	uterine hypertrophy

[a] Full references will be found in Stob (1983), from which this table is taken with permission.

Investigation of the metabolism of isoflavone oestrogens was stimulated by the problem of clover disease in sheep (Bennetts *et al.* 1946). Originally it was assumed that this condition, characterized by a marked loss of fertility, was due to the high levels of isoflavones present in subterranean, and other, clovers. Millington *et al.* (1964) were unable, however, to establish a relationship between the hormonal activity in sheep fed clover and the levels of genistein or biochanin A; a positive relationship was, however, found between the weaker oestrogen, formononetin, and such activity *in vivo*. It is now realized that the reason for this apparently anomalous situation lies in differences in the metabolism of these isoflavones in the digestive tract. Whereas biochanin A and genistein are converted into inactive products, formononetin is metabolized to the isoflavan equol (XVII), and it is this compound in the animal which produces the effect on oestrus (Shutt and Braden 1968). Equol does not, however, appear to be metabolized in the tissues of the sheep (Braden *et al.* 1967). The uterotropic effect of equol is only 10^{-3} that of 17β-oestradiol (Tang and Adams 1980), a potency which is consistent with its relative molar binding affinity to uterine cytosol receptor *in vitro* (Shutt and Cox 1972).

The major pathways which have been elucidated for the metabolism of formononetin (VIII) are shown in figure 4. The primary route, A, involves initial

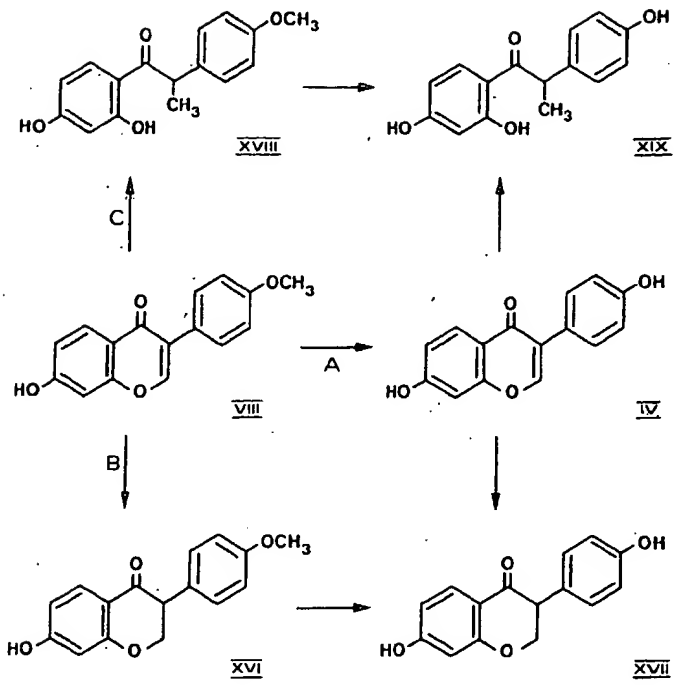


Figure 4. Metabolism of formononetin in the sheep.

demethylation (forming daidzein, IV) and then reduction. A secondary process (B) involves reduction to the 4′-methyl ether of equol (XVI) followed by demethylation. Equol possesses about one half of the affinity for binding to receptor sites of sheep uterine cytosol exhibited by genistein and approximately one quarter of the uterotropic activity of this compound when assayed by intravaginal tetrazolium reduction after oral administration to mice (Shutt and Braden 1968). About 70% of the formononetin ingested by sheep is converted to equol (Shutt *et al.* 1970) and, to a smaller degree, daidzein; in addition, other active metabolites, angolensin (XVIII) and O-desmethylangolensin (XIX) (figure 4, route C) may also be formed (Batterham *et al.* 1971). These compounds are uterotropic in mice and bind to sheep uterine cytosol receptor sites (relative affinities, angolensin 0·03 and O-desmethylangolensin 0·05) (Shutt and Cox 1972). In agreement with the findings of Bickoff *et al.* (1962), referred to above, the oestrogenic activity of the 4′-methyl ether, angolensin, was lower than that of its 4′-desmethyl analogue (Micheli *et al.* 1962).

In marked contrast to the above, biochanin A is metabolized in the sheep via demethylation (to genistein, V) and thence, by ring cleavage (presumably involving the intermediate phenyl-α-methylbenzyl ketone) to the oestrogenically inactive *p*-ethylphenol (XX, figure 5) (Braden *et al.* 1967).

Comparative studies in sheep and cattle revealed the latter to metabolize formononetin more rapidly and also to be more effective in conjugating isoflavones and their metabolites (Braden *et al.* 1971). In sheep the metabolism of biochanin A and genistein in the rumen is initially low but increases significantly over the first few days of grazing on clover and related forages; this is paralleled by a reduction in the hormonal effect of these crops. In marked contrast, the rate of formononetin degradation is not affected by time to any great extent, hence the pasture retains its oestrogenicity

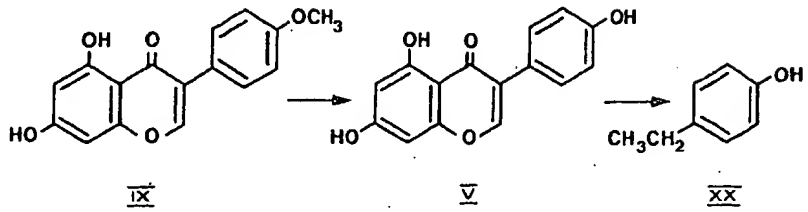


Figure 5. Metabolism of biochanin A in the sheep.

(Lindsay and Francis 1969). Provided that the livestock are removed from such pasture the oestrogenic effects are reversible. However, continued grazing will lead to permanent physiological changes of the reproductive tract (Lindner 1976). Equol has been identified in the urine of goats, rats and hens and in each case was considered to result from dietary isoflavone precursors, rather than being present in the diet *per se*.

Shutt *et al.* (1970) have observed the metabolism of isoflavones in sheep to proceed rapidly; for example, 1 g of biochanin A plus genistein was metabolized in about 90 min. Moreover, the data presented suggested that the initial demethylation (A in figure 4), rather than the reduction, was the rate-limiting step. Equol does not appear to suffer extensive degradation in the rumen, but is readily absorbed therefrom (residence time 1·7 h). There is a suggestion that residence times for isoflavones may be reduced under grazing conditions, a consequence of which may be the less complete metabolism of isoflavones in the rumen, a greater concentration of genistein resulting and/or a decreased production of equol from formononetin (Shutt *et al.* 1970). Consequently the oestrogenic activity, and effect, of such pasture in livestock depends upon the fine balance of isoflavone metabolism *in vivo*.

In contrast to the metabolites of steroidal oestrogens, isoflavones are readily conjugated as glucuronides and excreted. According to Shutt *et al.* (1967) circulating isoflavones are almost exclusively present in the form of biologically inactive glucuronides, although small amounts of the free compounds and their sulpho-conjugates, which can yield the free compounds *in vivo*, may also occur. A similar situation has been observed in man (Axelson *et al.* 1982). The plasma content of dietary isoflavones in sheep following the feeding of red or subterranean clovers was maximal 30 min after feeding and thereafter rapidly declined; the content of equol increased from 4 to 10 μg/100 ml plasma between 30 and 150 min after feeding, whilst the measured conjugated equol in plasma was very much higher (300–400 μg/100 ml) and was largely independent of feeding time (Shutt *et al.* 1967).

Equol was first reported in human urine by Axelson *et al.* (1982). Total daily excretion levels of two male subjects were 10·9 and 35·2 μg, whilst those of four female subjects ranged from 10·7 to 43·3 μg. In most cases $\geqslant$99·8% of the measured equol was excreted as the glucuronide, but in two subjects 5·7 and 9·9% was bound as the sulphoconjugate. Independently Adlercreutz *et al.* (1982) reported that there was no significant difference in the daily urinary excretion of equol by post-menopausal women who were vegetarians (mean 35·8 μg, range 0–113 μg), omnivores (mean 35·8 μg, range 0–102 μg) or suffering from breast cancer (mean 27·2 μg, range 0–74 μg). The maximum mean daily excretion measured was 565 μg over a three-day period, and at such a level the authors considered that a biological effect might result. Subsequently Bannwart *et al.* (1984) identified both daidzein and equol monoglucuronides in the urine of five female subjects. The levels found in four vegetarian subjects (two pre- and two post-menopausal) were much greater than that measured in the single pre-menopausal,

omnivorous subject (daidzein: average 396·6 μg/l, range 96·0–1108 μg/l compared with 21·6 μg/l; equol: average 4207 μg/l, range 1493–9663 μg/l compared with 46·0 μg/l). The variation in the vegetarian subjects was ascribed to differences in the composition of the diet. Apples, cherries, potatoes, garlic, hops and soya products were mentioned as the most probable sources of dietary oestrogenic compounds. A less important source of these compounds was considered to be products obtained from animals which had been fed oestrogen-containing forage. This seems probable, although only limited data is available upon which to base a judgement. According to Lindner (1967) the levels of such isoflavones accumulating in the adipose tissue of sheep (1 μg/g) was too low to present a serious health hazard. The effects of cooking and/or processing would, moreover, seem likely to reduce this figure further.

Recent work has emphasized the importance of soya as a source of dietary isoflavones (Axelson *et al.* 1984). Two healthy subjects were given 40 g of commercial texturized soya in place of meat, daily for 5 days. Urinary excretion of equol was found to increase 100–1000 fold (figure 6) and traces of daidzein glucuronides were also observed. Quantitatively similar results were observed in rats, approximately 100 μg of equol being excreted per gram of soya flour ingested. The figure for soya oil is much less (5 μg/g), indicating that little, if any, isoflavones are removed from soya during processing of the oil (see below). This result is of interest also since Vague *et al.* (1957) have reported cornification of the vaginal epithelium to occur in post-menstrual women following the administration of 100 g corn or olive oil per day for 10 days. The uterotropic effect of soya meal and soya-based rations in laboratory animals and poultry is well documented (Drane *et al.* 1980).

Setchell *et al.* (1984) have recently shown that certain people excreted little or no equol in the urine when fed 40 g of commercial soya protein daily for 5 days. The reasons for this behaviour are unclear, although it appears to be unrelated to the sex of the subject; the authors suggest that the rate of formation of equol was dependent upon dietary-related factors, such as the composition of the intestinal microflora, the intestinal transit time and variability in the redox level of the large intestine. These

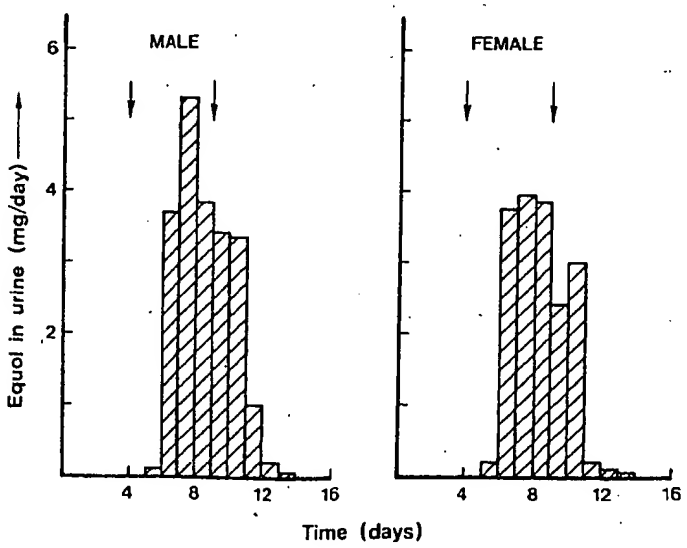


Figure 6. Daily urinary excretion of equol in humans (from Axelson *et al.* 1984). The arrows mark the period over which soya protein, 40 g/day, was fed.

workers also demonstrated that human faecal flora were able to degrade soya-rich broth components (presumably daidzin and daidzein) to equol. As the authors point out, it would be of dubious value to extrapolate the above findings, based upon six subjects, two of whom were obvious non-responders, to the population at large. These results do, however, emphasize the need for further study into the factors affecting phytoestrogen metabolism in man, the metabolic fate of dietary oestrogens in non-responders, and the variation in the rate of phytoestrogen metabolism in larger populations. The latter may in turn lead to the identification of particular 'at-risk' groups within the population at large.

Axelson and Setchell (1981) were unable to determine equol in the urine of germ-free rats fed a commercial, soya-containing ration. Glucosidases capable of converting isoflavone glycosides to the uterotropically active aglucones have been identified in man, as have enzymes which have been shown to carry out the conversion of daidzin to equol (Axelson *et al.* 1984). Although no evidence has yet been presented it would be expected that genistin→genistein→*p*-ethylphenol would represent a similar (but *de*toxifying) metabolic pathway in man.

The improvement of methods for the detection and quantification of isoflavones and their metabolites in plant material and biological samples has been of great importance in the development of an understanding of their chemical and biological properties. Such analysis has been effected by a variety of techniques, including paper chromatography (Markham 1975), thin layer chromatography (Beck 1964), gas chromatography (Naim *et al.* 1974), high-performance liquid chromatography, spectrophotometry, fluorimetry and immunoassay. Gas chromatographic methods, either alone or linked to mass spectrometry, have involved the prior derivatization of the molecules by converting free —OH groups to trimethylsilyl ethers or trifluoroacetyl esters (Naim *et al.* 1973). Gas chromatography-mass spectrometry procedures such as single ion monitoring have allowed very low levels of isoflavone metabolites to be measured (Bannwart *et al.* 1984, Axelson *et al.* 1984), and using conventional gas chromatography procedures the former authors have demonstrated a detection limit of 1 μg equol/24 h urine sample. With such low levels, the isolation, extraction and concentration of the compound(s) of interest from the bulk sample is of paramount importance. The use of reversed-phase silica for preliminary clean-up has proved especially useful for the extraction of isoflavones and equol (and their conjugates) from urine, following which enzyme hydrolysis and ion exchange clean-up processes are employed (Axelson *et al.* 1984, Bannwart *et al.* 1984). Use of DEAE Sephadex (base form) enables a degree of separation between mono- and diphenolic species to be effected (Axelson *et al.* 1982).

The advantage of high-performance liquid chromatography techniques is that the samples can be examined without the need for derivatization; under such conditions both free compounds and conjugates may be analysed directly. Following the original report of Kallela and Saastamoinen (1978), a number of techniques have been described, which almost invariably use reversed-phase systems. Methods developed for the analysis of isoflavones in clover and other fodder crops usually rely upon the facile hydrolysis of the glucosides during plant maceration and extraction such that the isoflavone aglucones are separated and quantified. Petterson and Kiessling (1984) and Sachse (1984) both include chemical hydrolysis prior to sample analysis. Free isoflavones and glucosides are readily determined in soya by high-performance liquid chromatography and, of the methods described, the present authors favour that of Eldridge (1982a) in which all of the likely soya isoflavones are separated, an internal

standard is included and no problems of co-eluting impurities are encountered. The latter severely limits the usefulness of a semi-preparative method for the isolation of daidzin and genistin, reducing the loading capacity to an extent that conventional chromatography (using Sephadex LH20) was of comparable efficiency (Farmakalidis and Murphy 1984a).

The analysis of soyabean (meal) and fractions have almost invariably revealed the presence of daidzin, daidzein, genistin, genistein, glycitin-7β-glucoside and glycitein, the latter two being uterotropically inactive and for this reason not included in table 3. Small amounts of formononetin were also claimed to be present by Shemesh *et al.* (unpublished, cited in Lindner 1976), but details of the method were not given; in the absence of any independent confirmation and bearing in mind the obvious differences between the results of these workers and others (table 3) for the levels of the other isoflavones, this report should be treated with caution. It is generally held that the major proportion of soyabean isoflavones are present as glucosides (table 3), but as has been indicated these are readily degraded by intestinal bacteria prior to metabolism, conjugation and excretion. Bickoff *et al.* (1962) have reported that 8 mg of genistein (or 10 mg of daidzein) was the minimum dose needed to induce a hormonal response in mice; hence the oestrogenic effect of soyabean meal and soyabean-containing commercial rations on poultry and laboratory animals is readily understood, especially when it is further realized that biologically significant levels of coumestrol and its methyl ethers may also be present.

Much research on the isoflavones of pasture and forage crops has demonstrated that many factors (e.g. the physiological age of the plant, its genetic origin, climatic and environmental factors associated with growth) can affect the ultimate content of these compounds in the plant (Bickoff 1968, Rossiter and Beck 1967), and more recent work has shown these factors also to be important in soya. However, additional consideration must be taken of the effect of subsequent processing, especially as it relates to human food ingredients.

Eldridge and Kwolek (1983) have shown that the defatting of full-fat soya does not remove isoflavones or their glucosides, contrary to the earlier claim of Booth *et al.* (1960). Support for this later finding comes from the work of Axelson *et al.* (1984) referred to above. Analysis of soyabean hull (8% by weight), hypocotyl (2%) and cotyledon (90%) fractions revealed isoflavone contents of 10–20 mg/100 g, 1405–1750 mg/100 g and 319–808 mg/100 g respectively. It should be noted that coumestrol is concentrated primarily in the hull and testa portions (Lookhart 1979). Daidzin and glycitin account for more than 95% of the total isoflavone content of the hypocotyl, whereas in the cotyledon the latter is almost absent and genistin predominates. Eldridge (1982b) found that soya protein concentrate (containing 70% protein) prepared by aqueous leaching contained higher levels of isoflavones (247 and 317 mg/100 g) than were present when an aqueous alcohol process was used (16 and 43 mg/100 g). Soya protein isolates, containing 90% protein, although obtained by a variety of unspecified procedures, contained similar isoflavone contents (103–145 mg/100 g), most of which was genistin and genistein. Combined levels of daidzin and daidzein, yielding equol on metabolism, ranged between 24 and 51 mg/100 g. Seo and Morr (1984) found a commercial protein isolate to contain 96 mg isoflavones/100 g. Whilst in general agreement with these findings, Murphy *et al.* (1982) observed the level of isoflavone glucosides in soyabeans to decrease substantially on germination, during protein isolation or when calcium-precipitated tofu was prepared. There appeared, however, to be no corresponding increase in the free forms of these isoflavones. According to György *et al.* (1964)

Table 3. Oestrogenic isoflavone content of soya and its products.

Sample	Daidzin (mg/100 g)	Daidzein (mg/100 g)	Genistin (mg/100 g)	Genistein (mg/100 g)	Formononetin (mg/100 g)	Reference
Soyabean meal	62	48	127	40		Eldridge (1982a)
Soyabean meal	11·7, 0	0, 2·2	74·7, 102·4	4·0, 2·4		Murphy (1982)
Soyabean meal	56·7, 56·1	4·9, 14·5	65·5, 81·3	9·7, 18·7		Pettersson and Kiessling (1984)
Soyabean meal	42	17·8	151	108		Pratt and Birac (1979)
Soyabean flakes	59·6 ± 8	5·6 ± 0·7	215 ± 9	6·7 ± 8		Seo and Morr (1984)
Soyabean flour	48–77	8–48	58–154	4–46		Eldridge (1982)
Soyabean cake		30 ± 5		18·6 ± 2·7	4·3 ± 2	Shemesh *et al.* (in Lindner 1976)
Soyabean flakes	114	2·5	188·5	4·4		Eldridge and Kwolek (1983)
Soya-based animal ration	7		42–45	7		Murphy *et al.* (1982)

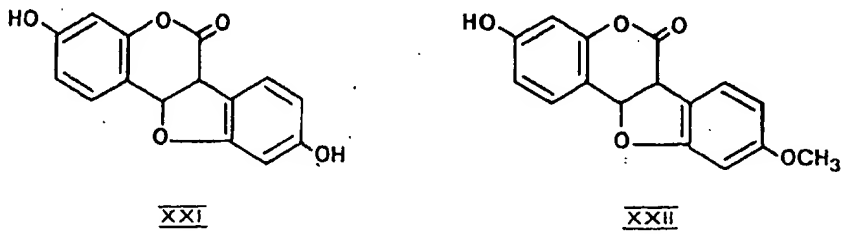
daidzin and genistin are hydrolyzed by *Rhizopus oryzae* during the fermentation of soyabeans to produce tempeh. Defatted soya flakes contained 287 mg isoflavones/100 g (Seo and Morr 1984) and this was decreased by various protein isolation procedures to 203 mg/100 g (acid precipitation), 53 mg/100 g (dialysis), 8·3 mg/100 g (ion exchange) and 6·1 mg/100 g (activated charcoal treatment). A commercial sample of soya protein hydrolysate contained genistein and daidzein contents of 54 and 15·2 mg/100 g, respectively; animal rations containing soya hydrolysates were also observed to possess very low levels of isoflavones (Murphy 1982). Germinated bengal gram (*Cicer arientanum*) was found to contain biochanin A and formononetin at levels of 71 and 77 mg/100 g (Dziedzic and Dick 1982) and 98·6 mg and 44·1 mg/100 g (Sharma 1979a), respectively. The latter worker also identified daidzein (5·1 mg/100 g).

Bartholomew and Ryan (1980) found daidzein, genistein, formononetin and biochanin A all to be non-mutagenic when screened using the *Salmonella*/mammalian microsome assay, the behaviour of the first two compounds being in agreement with the findings of Sugimura *et al.* (1977), and confirmed by Murphy and Glatz (in Murphy 1982).

Isoflavone aglucones have been shown to be responsible in part for the antioxidant activity of soyabeans and their products (György *et al.* 1964, Pratt and Birac 1979, Pratt *et al.* 1981). These compounds also contribute to the astringent and bitter tastes of defatted soyabean (How and Morr 1982) and soy protein products (Huang *et al.* 1981). Soyabean isoflavones possess marked antifungal activity, whereas the glucosides are almost without action (Naim *et al.* 1974). Sharma (1979b) has demonstrated that biochanin A, formononetin and pratensein possess hypolipidaemic activity in the albino rat, but daidzein (and genistein (Ollis 1962)) was inactive. It was considered that this, at least in part, explained the hypocholesterolaemic activity of the black gram and navy bean (Saraswati Devi and Kurup 1972, Hellendoom 1976).

Coumestans

Coumestans possess structures exhibiting close similarity to those of isoflavones to which they are biosynthetically related. A relatively large number of these compounds have been isolated from plants (Wong 1975), but only a few have been shown to possess uterotropic activity. For example, Verdeal and Ryan (1979) list eight coumestans which have been identified in alfalfa, only two of which possess such activity. These compounds, coumestrol (7,12-dihydroxycoumestan, XXI) and 4′-methoxycoumestrol (7-hydroxy-12-methoxycoumestan, XXII) (figure 7) are the most common of this class of oestrogen and have been reported in alfalfa, ladino clover and other fodder crops where their presence is associated with widespread problems of animal performance (Stob 1983). According to Hanson *et al.* (1965), over 90% of the oestrogenic activity of potent dehydrated alfalfa samples was due to its coumestrol content and Lookhart



XXI XXII

Figure 7. Structure of coumestans.

(1980) has found serious oestrogenic effects to result from feeding cattle haylage containing 37 mg coumestrol/kg. Bickoff *et al.* (1960) and others (Micheli *et al.* 1962) have investigated the effect of structural changes in the coumestan molecule on its hormonal activity. Phenolic groups in the 7,12 positions were important; thus the 7-methyl ether and 12-methyl ether (XXII) possessed only 54% and 15%, respectively, of the uterotropic activity of coumestrol itself when administered orally to mice. 7,12-Diacetoxycoumestrol was almost as active as the parent compound when administered in the same manner, presumably reflecting the lability of the acetoxy groupings *in vivo*.

As may be seen from figure 1, the uterotropic potency of coumestrol in the mouse is greater than that of the isoflavones and, as with the latter, variation occurs according to species and means of administration. Braden *et al.* (1967) found coumestrol (administered intraruminally) to be 15 times more active than the most potent isoflavone and it is even more potent when injected intramuscularly. In the mouse, coumestrol is 35 times more active (and its diacetate 24 times more active) than genistein, but still possesses less than 0·03% of the activity of diethylstilboestrol (Bickoff *et al.* 1962). The coumestans, like the isoflavones, bind competitively to mammalian oestrogen receptor sites and are more active when assayed in this manner. The relative binding efficiency (that of 17β-oestradiol = 100) of coumestrol has been reported as 1·4 (rabbit uterine cytosol, Shemesh *et al.* 1972), 4·9 (sheep uterine cytosol, Shutt and Cox 1972), 4·9 (rat uterine cytosol, Verdeal *et al.* 1980) and 19·7 (calf uterine cytosol, Lee *et al.* 1977). When tested in human cancer cell preparations the relative affinity of coumestrol was measured as 9·8 (Martin *et al.* 1978). According to Fredericks *et al.* (1981) coumestrol may exert its effect on fertility *in vivo* by inhibiting follicle stimulating hormone. Little is known about the metabolism of coumestrol; Kelly (1972) has found the compound to be rendered less active in sheep over a period of 7–14 days. Whilst this might be due to the formation of less active metabolites, the chemical nature of which is obscure, more recent work suggests an alternative explanation. Coumestrol is conjugated *in vivo*, but to a rather lower extent than the isoflavones. Thus Kelly and Lindsay (1978) found between 20% and 40% of the total coumestrol in sheep's plasma to be present in the free form (compared to less than 10% in the case of the isoflavones) (Shutt *et al.* 1967). Significantly the concentration of free coumestrol in sheep's plasma remained constant over 16 days, during which time the animals became biologically less sensitive to the oestrogenic effects of this compound. The loss of sensitivity, moreover, appeared to be related to the amount of dietary coumestan and the period of exposure. Further work is needed to clarify the factors underlying these interesting observations. The biological effects of administering coumestrol to animals is shown in table 4.

Coumestrol has been found in a range of plant products commonly consumed by man (table 5). The highest levels were noted in sprouts of alfalfa and, especially, soyabean (Knuckles *et al.* 1976b). Legume sprouts and shoots have in recent years been consumed in increasing amounts by certain sections of the population of the UK and other western countries. It would seem prudent to conduct a more detailed study of the coumestrol (and isoflavone) contents of these materials using modern analytical methods. In the aforementioned work, Knuckles *et al.* used paper chromatography allied to fluorimetric detection and quantification (Knuckles *et al.* 1976a); at the present time, however, the best method of analysis would appear to be high-performance liquid chromatography (Lookhart *et al.* 1978, 1980) using ultraviolet or fluorimetric detection. By judicious choice of mobile and stationary phases it is also possible to monitor isoflavones and coumestrol simultaneously (Pettersson and Kiessling 1984).

Table 4. Effects of pure coumestans and zearalenone.[a]

Animal	Compound	Dose	Effect
Mouse	coumestrol	100–500 μg/g diet	uterine hypertrophy
	coumestrol	500 μg/g diet	antigonadotropic
Rat	coumestrol	1 mg injected, 5 days neonatally	persistent oestrus syndrome
	coumestrol diacetate	125 μg injected	increased protein and phospholipid synthesis in uterus
Sheep	coumestrol	12 mg injected, 1·4 g intraruminally	uterine hypertrophy
Mouse	zearalenone	10 μg/g diet	uterine hypertrophy
		20 μg injected	uterine hypertrophy
Rat	zearalenone	1 mg, oral	uterine hypertrophy
		600 μg topical to skin	uterine hypertrophy
Swine	zearalenone	1–50 mg daily, oral	hypertrophy vulva, vagina, uterus and mammae; metaplasia of cervical epithelial cells
		100 μg/g diet	infertility
		25–100 μg/g diet	infertility, nymphomania, pseudopregnancy, reduced litter size, smaller pigs, malformations, juvenile hyperoestrogenism, probable fetal resorption
Chicken	zearalenone	300–800 μg/g diet	hypertrophy of vent, oviducts and cloacal bursa, eversion of cloaca
Turkey	zearalenone	300–800 μg/g diet	hypertrophy of vent, oviducts and cloacal bursa, eversion of cloaca
Monkey	zearalenone	14 or 56 μg/kg injected	stimulation, LH[b] surge
		14 μg/kg injected	serum LH depression
		400 μg daily, orally for 4 days	serum LH depression

[a] Full references will be found in Stob (1983), from which this table is taken with permission.
[b] LH = luteinizing hormone.

Table 5. Coumestrol content of plant products.[a]

Product	Coumestrol content (μg/100 g dry weight)
Alfalfa sprouts (fresh)	500
Soyabean sprouts (fresh)	7110
Soyabeans (dry)	120
Defatted soyabean meal (dry)	40
Soyabean concentrate	20
Soyabean isolate	60
Frozen green beans	100
Frozen snow beans	60
Frozen green peas	40
Frozen Brussels sprouts	40
Dried red beans	40
Dried split peas	30
Frozen spinach leaf	10

[a] Data from Knuckles *et al.* (1976) with permission.

The coumestrol content of plant material has been observed to vary with a variety of factors (Bickoff *et al.* 1969). For example, Hanson *et al.* (1965) have shown that of alfalfa to be affected, to various degrees, by variety, stage of growth, cutting, the year and location and, to a significant degree, by the presence of disease. Coumestrol has been observed to accumulate in alfalfa and other legumes following insect (Loper 1968) or fungal attack (Loper 1968, Loper and Hanson 1964, Stuthman *et al.* 1966, Loper *et al.* 1967). According to Sherwood *et al.* (1970) coumestrol was not translocated from the infected area to other parts of the plant. Whereas coumestrol in undamaged, non-infected plants was metabolized via the isoflavone pathway (Grisebach and Barz 1963, 1964), the origins of the coumestrol biosynthesized as a result of such insect or fungal damage is unknown.

Concern over the presence of coumestans in alfalfa and ladino clover has resulted from the reduced reproductive performance of animals maintained on such fodder (Hanson *et al.* 1965, Bickoff *et al.* 1969) and both breeding programmes and improved husbandry practices have been initiated to reduce the extent of the problem. Of the latter, treatment with agrochemicals can minimize the pest and fungal attack which results in accumulation of coumestrols and other plant phenolics; moreover, the intake of coumestrol by animals can also be reduced by the feeding of immature plants in which the coumestrol content is known to be lower than in the mature plant. In the absence of any information concerning the amount, if any, of coumestans which enter the human body indirectly via the residues in animal products and milk obtained from livestock grazing on oestrogenic pasture, concern over the intake of these compounds by man is centred mainly upon their presence in common food plants (table 4), vegetable protein and 'health' products.

Leaf protein concentrate has been suggested as a source of protein for humans, and methods have been described for its preparation from alfalfa (Kohler *et al.* 1968, Edwards *et al.* 1975). The effect of such processing on the coumestrol content has been examined by Knuckles *et al.* (1976b). Relatively little of the original coumestan content of the alfalfa (11–118 mg/kg) was removed in the solubles during the early stages of the processing. Protein concentrates possessing 9–14 mg coumestrol/kg were obtained by commercial-type processing in which heat coagulation and washing was carried out under acid conditions (pH 4·5–6·5), whereas if the medium was kept alkaline (pH 8·5–9·5) the coumestrol content was much lower (3 mg/kg) due to the greater solubility of the oestrogen under these conditions. Diafiltered alfalfa leaf protein concentrate possessed a coumestrol content of only 0·4 mg/kg (measured as freeze-dried powder). Since the coumestrol content of diseased or damaged alfalfa leaves may exceed 1000 mg/kg, i.e. 10–100 times that of undamaged tissue, it is clearly important that the quality of the materials selected for processing be maintained as high as possible.

Alfalfa and other leguminous products have been widely marketed in recent years as health foods, tonics and supplements. Recently, Elakovich and Hampton (1984) have analysed commercial alfalfa tablets and found these to contain 20–194 μg coumestrol/g, equivalent on a daily dosage basis to 1–2 mg of coumestrol. The effect of long-term exposure to such levels (together with that of any isoflavone oestrogens which may also be present) cannot yet be ascertained. However, this work clearly points to the desirability of monitoring the contents of physiologically active substances in health products since the 'recommended' doses (if stated) are frequently exceeded and the products may not be covered by the same legislative controls as foods and feeding-stuffs.

Coumestrol has been observed to possess tumour-promoting activity similar to that of 17β-oestradiol and diethylstilboestrol for dimethylbenzanthracene-induced rat mammary tumours (Verdeal *et al.* 1980). However, Bartholomew and Ryan (1980) have reported this compound to be non-mutagenic in the Ames test. Both coumestrol and its 4′-methyl ether had been shown to possess weak antifungal activity (Van Etten 1976).

Resorcylic acid lactones

Unlike the previous two groups of plant oestrogens, the resorcylic acid lactones are not intrinsic components of food plants but are secondary mould metabolites of fungal species, principally *Fusarium*, e.g. *F. roseum* var. *graminearum* (*Gibberella zeae*) which are common field organisms which also proliferate in poorly stored grains, oil seeds and hay (Caldwell *et al.* 1970, Eugenio *et al.* 1970, Sherwood and Peberdy 1972, Abbas *et al.* 1984). There have been a number of detailed reviews on the chemistry (Shipchandler 1975), production and biological activity (Mirocha *et al.* 1971, 1977, Mirocha and Christensen 1974, Pathre and Mirocha 1976, Hidy *et al.* 1977, Betina 1984) of these compounds and a comprehensive coverage of these and other aspects of *Fusarium* moulds is now available (Moss and Smith 1984). The economic losses associated with the feeding, especially to swine and cattle, of rations containing such mould-damaged produce have rightly meant that emphasis is primarily placed on the effects of such compounds on livestock, rather than on humans. However, since there is, at least in principle, the possibility of these compounds being carried over into humans via the consumption of animal products, and as many grain and cereal products are now formulated directly for human consumption, it is appropriate to consider the levels of such compounds likely to enter the human body and, thereby, assess the likely risk from such compounds.

The most common oestrogen of this group is zearalenone (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl) β-resorcylic acid lactone, XXIII). The reduced compound, zearalanol (XXVI, figure 8), has been marketed as a growth promoter in sheep and bovines. The main metabolites of zearalenone are the epimeric β- and α-zearalenols (XXV and XXIV). Other compounds have been identified (Verdeal and Ryan 1979), but in general little is known about their biological activity. Zearalenone is usually described as a mycotoxin (F-2 toxin) but some reviewers consider this as inappropriate (Stob 1983). There have been a number of cases reported where the feeding of *Fusarium*-infected rations have caused death, abortion and other serious physiological disorders in livestock and poultry, and in some cases these were attributed to the presence of zearalenone. It is possible that other, more toxic, substances were also present, since such moulds normally produce a number of mycotoxins simultaneously; these may include trichothecenes, such as T-2 toxin, deoxynivalenol and diacetoxyscirpenol. As Stob (1983) has stated, the involvement of zearalenone in some of the more distressing symptoms associated with the feeding of *Fusarium*-infected rations should be treated with circumspection and the role of zearalenone itself should be demonstrated in controlled feeding trials, where such additional compounds can be excluded. The observed LD_{50} of zearalenone is certainly far removed from those of other mycotoxins, being 5, 10 and 20 g/kg in female guinea pigs, rats and mice respectively. For these and other reasons, Stob (1983) has suggested the terms 'mycoestrogen', 'fungal oestrogen' or 'oestrogenic metabolite' as being more appropriate.

Zearalenone, zearalenol and zearalanol have been found to bind to mammalian

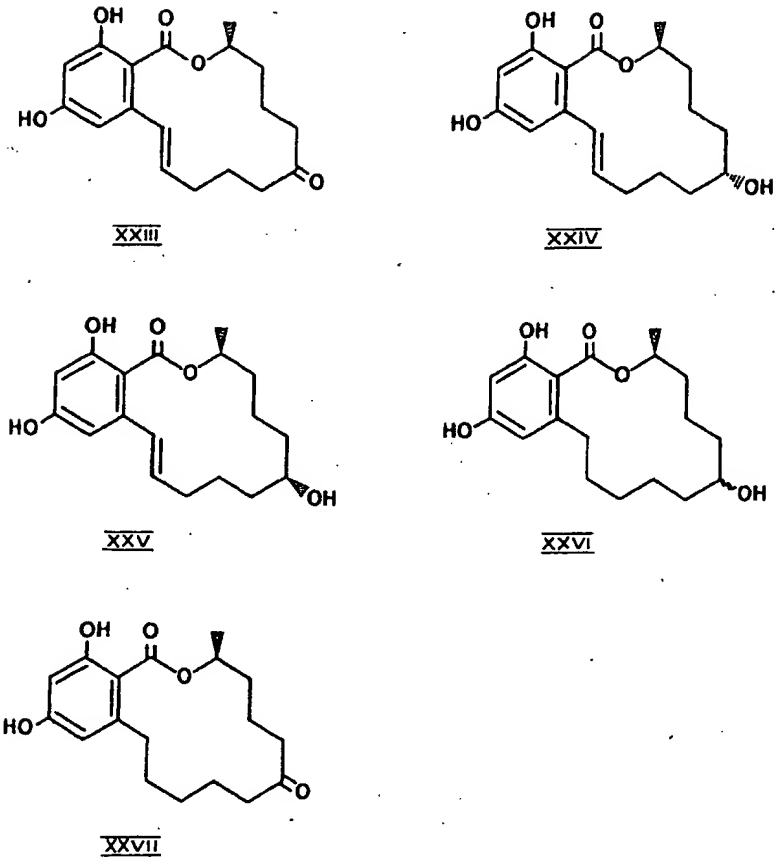


Figure 8.

oestrogen receptor sites. Kiang *et al.* (1978) showed these to bind to uterine cytosol and nuclear receptors in the order *cis*-zearalenone (not naturally occurring) > *trans*-zearalenone > zearalenol (stereochemistry unspecified) > zearalanol. All four compounds almost completely inhibited the binding of 17β-oestradiol at a ratio of 100:1. Katzenellenbogen *et al.* (1979) found α-zearalenol to be more active than either β-zearalenol or zearalanone when measured by competitive or direct binding assays using rat uterine cytosol receptors. The former compound was observed to possess 13·6% and 15% of the effect of 17β-oestradiol upon competitive and direct binding analysis, respectively.

It has been suggested (Ueno and Tashiro 1981) that the oestrogenic effect of zearalenone is due to its metabolism to zearalenol, and this suggestion has been supported by more recent work (Sheehan *et al.* 1984). Despite the structural dissimilarity between zearalenone and 17β-oestradiol, as Duax *et al.* (1984) have pointed out, there is considerable similarity between their respective hydrophobic bulk. Zearalenone binds to rat hepatic cytosol oestrogen receptors (Powell-Jones *et al.* 1981) as well as to those of rat uterus, with which it has been found to bind more strongly than the isoflavones but less strongly than coumestrol (Verdeal *et al.* 1980). Radio-labelled zearalenone, injected intravenously into mice, was found to be bound to oestrogen target organs, e.g. uterus, intestinal testicular cells and ovarian follicles (Appelgren *et al.* 1982). Studies by Martin *et al.* (1978) showed that zearalenone was less potent than

either isoflavones or coumestrol when assayed by competitive binding to human breast cancer cell oestrogen receptors. The uterotropic activity of zearalenone has been demonstrated by various workers (Stob 1983)—for example, when administered by mouth it was 10^3 times less active in the mouse than was diethylstilboestrol (figure 9). By subcutaneous injection in the same species, the compound was 500 times less active than 17β-oestradiol (Katzenellenbogen *et al.* 1979). Mirocha *et al.* (1978) have shown *cis*-zearalenone to possess stronger uterotropic activity than the natural *trans*-isomer; *cis*- and *trans*-zearalenols were found to be of comparable activity by the same workers.

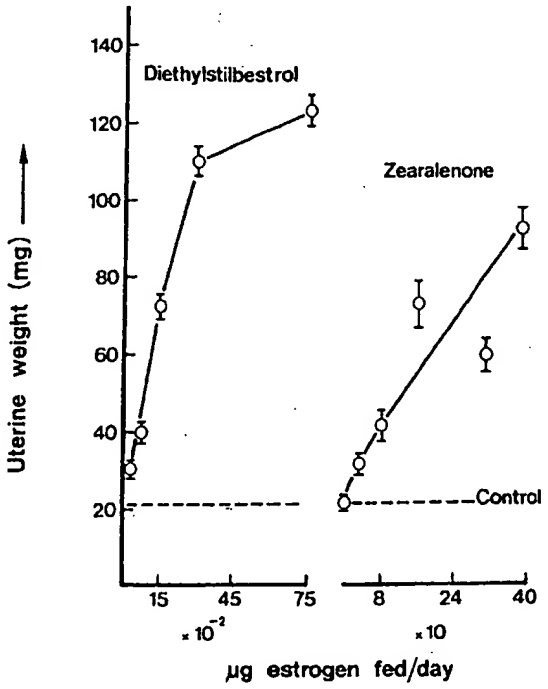


Figure 9. Relative uterotropic activity of orally administered diethylstilboestrol and zearalenone (after Stob 1983).

The biological activity of zearalenone in animals is shown in table 4. Data on the oestrogenic potency of zearalenone in rats has been obtained by Kumagai and Shimizu (1982); by uterotropic assay this compound possessed less than 0·1% of the activity of 17β-oestradiol, a figure in agreement with that resulting from estimation of vaginal cornification following systemic administration. In contrast, the vaginal cornification bioassay indicated that zearalenone possessed about 1% of the activity of 17β-oestradiol when administered locally. Furthermore, neonatal exposure to zearalenone produced anovulatory sterility in the rat, the potency being 10% that of 17β-oestradiol. There is considerable evidence that pigs are especially sensitive to zearalenone (Mirocha *et al.* 1974, Chang *et al.* 1979) with hormonal effects resulting from as little as 1–5 mg/kg diet. According to Chang *et al.* (1979), the inclusion of 25–100 mg zearalenone/kg in the ration of sows led to multiple reproductive deficiencies, including infertility, reduced litter size and weight and hyperoestrogenism. Zearalenone and zearalanol have been shown to increase weight gain when implanted subcutaneously in sheep (Hidy *et al.* 1977) and subsequent study demonstrated the latter to be especially

effective in steers and heifers (Willemart and Bouffault 1983). Further work also resulted in synthetic zearalanol implants being produced commercially.

In primates, orally administered zearalenone was 80 or 160 times less active than 17β-oestradiol or diethylstilboestrol, respectively, in inhibiting synthesis and release of gonadotropins (luteinizing hormones) from the anterior pituitary (Hobson *et al.* 1977), but the effect is much greater if the compounds are administered by subcutaneous injection and all three compounds are then of comparable effectiveness. In humans, 75–100 mg of zearalenone/day have been reported to be effective in the treatment of post-menopausal syndrome in women (Utian 1973, Hidy and Baldwin 1976b) and, according to Hidy *et al.* (1977), the clinically effective daily dose of zearalanol for such purposes is 50–75 mg, a practical result which was in agreement with that inferred from animal experiments. Both zearalanol and zearalenone are effective as oral contraceptive agents in humans (Hidy *et al.* 1976a).

According to unpublished work cited by Hidy *et al.* (1977), the major metabolite of orally administered zearalenone in the sheep was β-zearalenol (XXV) which was shown to possess only 25% of the oestrogenic activity of the parent compound. Ueno and Tashiro (1981) observed only small amounts of this metabolite in rat faeces, the main product being the epimer, α-zearalenol (XXIV), which was three times more uterotropically active than zearalenone and also bound more strongly to mammalian uterine cytosol receptor. α-Zearalenol may be present in both the free and conjugated (β-glucuronate) forms (Kiessling and Pettersson 1978, Olsen *et al.* 1981) and conjugation of zearalenone itself may also represent a significant detoxification process (Kiessling and Pettersson 1978, Olsen *et al.* 1981) in the rat. Rumen microbes have been observed to metabolize zearalenone into α- (mainly) and β-zearalenols and it has been suggested that an additional explanation of the oestrogenic effect of zearalenone-zearalenol metabolism is the interruption of normal steroid metabolism via the necessary involvement of hydroxysteroid dehydrogenase (Kiessling and Pettersson 1978, Olsen *et al.* 1981). As has been mentioned earlier, zearalanol has been used to improve weight gain of livestock. The main metabolite of this compound in a wide range of species, including man, is zearalanone (XXVII); both compounds have been found in the free and bound forms (Hidy *et al.* 1977).

According to Dixon and Russell (1983), when four cattle were implanted with 36 mg of zearalanol, mean maximum urine levels were 13·5 μg/l (22 days after implantation) and declined to 2·9 μg/l by day 69. Two experiments were conducted with sheep, who received 12 mg zeralanol implanted into the base of the ear. Mean maximum urine levels were reached after 35 days (45 μg/l) and 56 days (90 μg/l) in the two experiments, and thereafter declined to 26 μg/l (day 42) and 11·7 μg/l (day 70), respectively. Ruddick *et al.* (1976) reported zearalenone to be teratogenic although more recent work (Davis *et al.* 1977, Wardell *et al.* 1982) could not confirm this.

Numerous methods have been described for the analysis of zearalenone (Gilbert 1984), including thin layer and paper chromatography (with colourimetric or ultraviolet detection) (Caldwell *et al.* 1970), gas chromatography (utilizing the trimethylsilyl- or pentafluoropropionate derivatives) (Steele *et al.* 1976, Holder *et al.* 1977), gas chromatography-mass spectrometry (Mirocha *et al.* 1974, Scott *et al.* 1978) and high-performance liquid chromatography (Scott *et al.* 1978, Cohen and Lapointe 1980). Thin layer and high-performance liquid chromatography have also been used to separate zearalenone and its metabolites in biological samples (Kiessling *et al.* 1984, Ueno and Tashiro 1981). The detection limit for zearalenone using high-performance liquid chromatography, with Spherisorb 5 μm column and fluorescence detection, was

5 μg/kg in corn flakes (although a second high-performance liquid chromatography column was needed to remove an interfering compound in other corn products) and 10 μg/kg in corn (Scott *et al.* 1978, Ware and Thorpe 1978, respectively). For purposes of routine screening, simpler techniques using thin layer chromatography have been developed. With Fast Violet B as spray reagent, detection limits of 20 μg/kg (Scott *et al.* 1978) and 80 μg/kg (Swanson *et al.* 1984) have been reported for zearalenone in corn and corn-based foods. The latter workers also considered the method amenable for the qualitative, but not quantitative, screening of zearalenol (detection limit 200 μg/kg). Immunological techniques have been developed for the detection and quantification of zearalanol (Dixon 1980, Dixon and Russell 1983, Thouvenot and Morfin 1983) but since related compounds, such as zearalanone and zearalenone, may possess significant cross-reactivity towards the antiserum, a preliminary separation with high-performance liquid chromatography has been recommended (Jansen *et al.* 1984).

The extent of the contamination of grain crops with *Fusarium* species may be considerable. In 1972, 38 out of 223 corn samples from areas in the USA where such contamination was suspected or expected were found to contain zearalenone, the levels ranging from 100 to 5000 μg/kg (Eppley *et al.* 1974). A similar study the following year revealed zearalenone levels of 38–294 μg/kg in 19 out of a total of 315 marketable corn samples (Stoloff *et al.* 1976). There was clear evidence of localized regional occurrence with 10% of the samples from the Corn Belt (17 out of 169) being affected. The same workers also measured zearalenone contents of 97–10 400 μg/kg in 57 samples of obviously damaged corn. The results of other surveys of wheat, grain sorghum, soyabeans and corn have been summarized by Bennett and Shotwell (1979). Zearalenone has been detected in six samples of Mexican corn intended for human consumption, but the levels were not quoted (Mirocha *et al.* 1972). Of 293 samples of the 1982 Australian maize crop recently examined (Blaney *et al.* 1984), 85% contained zearalenol; the mean concentration was 170 μg/kg but four samples possessed in excess of 1000 μg/kg. Côté *et al.* (1984) found 40 out of 342 feed samples, obtained in 1981 from the area around Illinois and suspected of causing or contributing to animal health problems, to contain zearalenone. Levels ranged from 100 to 8000 μg/kg, with a mean of 660 μg/kg. In Canada, problems associated with *Fusarium* infection of corn and other crops would seem to occur predominantly in Ontario (Andrews *et al.* 1981). Analysis of suspected samples over the period 1972–1977 revealed some 10% (214 out of 2022) to possess zearalenone, levels ranging from 10 to 141 000 μg/kg, the mean being 3850 μg/kg. Zearalenone, deoxynivalenol and, apparently for the first time, aflatoxin B_1, have recently been identified in commercial wheat samples from the mid-western USA. Of a total of 33 samples examined, zearalenone was present in trace amounts in two samples and, in another three, at levels of 35, 90 and 115 μg/kg (Hagler *et al.* 1984).

There is some disagreement over the effectiveness of chemical treatments for detoxification of zearalenone-contaminated grain. An American patent (Tamas and Wöller 1977) describes either 3–6% aqueous hydrogen peroxide or ammonium hydroxide as effective, but the removal of zearalenone was not quantified. However, unpublished work, referred to by Bennett and Shotwell (1979), found the ammoniation process used for removal of aflatoxins to have no effect on zearalenone levels. More recently, Kallela and Saastamoinen (1981) have shown the farm grain preservative 'Gasol' to have a beneficial effect in reducing the levels of zearalenone in stored grains.

A considerable amount of the world grain crops is used as human food sources, either directly or after processing. In many parts of the world such use represents the

major part of the crops' utilization. There have been a number of reports of zearalenone being found in southern African foods, drinks and raw materials. Thus levels of 100–800 μg/kg were measured in corn used for the brewing of Zambian beer (Lovelace and Nyathi 1977) with an average of 920 μg/kg (maximum 4600 μg/kg) being found in such beers and 800–4000 μg/kg in the corn malt used in the brewing process. Of 55 samples of sour drinks, porridges and beers from Swaziland, six were found to contain zearalenone (referred to in Bennett and Shotwell 1979) at levels between 800 and 5300 μg/kg. Of local beers from Lesotho, 12% of the 140 samples examined also contained this oestrogen (300–2000 μg/kg). Rather lower levels were found in Lesotho beer by Martin and Gilman (1976) (approximately 50 μg/kg) and samples of maize porridge, sorghum malt were also found to be contaminated (Martin 1974). MacDonald and Raemakers (1974) found zearalenone in South African maize samples. Together with zearalenone, the presence of other, more toxic, metabolites may be expected (Bennett and Shotwell 1979) and although the climate in southern Africa might be expected not to be such as to facilitate such mould growth as might occur in other parts of the world, Marasas *et al.* (1977) have found strains of *F. graminearum* in southern Africa capable of producing deoxynivalenol, and possibly other mycotoxins.

According to Stoloff and Dalrymple (1977), zearalenone was not detected in the primary or by-products from dry milling operations. Bennett *et al.* (1976, 1978) have examined the effects of processing on naturally contaminated corn. Wet milling was found to concentrate the oestrogen in the gluten fraction with lesser amounts being found in the milling solubles, fibre and germ respectively. The starch fraction was free of zearalenone. Dry milling led to a two- to three-fold concentration of the zearalenone in the germ. Both milling processes led to a concentration of the zearalenone into fractions used as animal feedingstuffs.

Scott *et al.* (1978) have examined various corn products for zearalenone using both high-performance liquid chromatography and gas chromatography. Largest amounts were found in a sample of cornmeal (26 μg/kg), although two other samples contained no detectable amounts. Frozen corn contained 2 μg/kg, corn chips 0 and 2 μg/kg, popcorn 0 and 7 μg/kg and three samples of cornflakes 0, 0·4 and 14 μg/kg respectively. The carry-over of zearalenone in cattle consuming naturally infected wheat rations has been studied by Shreeve *et al.* (1979). Concentrates (385–1925 μg zearalenone/kg) were fed to two cows for 7 weeks. No zearalenone (detection limit 4 μg/kg) residues were detected in muscle, kidney, liver, serum, milk or urine. The result should be interpreted with some caution bearing in mind the number of animals used and the inability of the analytical method used to detect zearalenone metabolites. The study also revealed apparent indications of interactions between dietary fungal metabolites which would warrant further examination. Mirocha (1981) has detected α- and β-zearalenol (16–76 μg/kg) in the milk of a cow following the oral dosage of [^{3}H]zearalenone. Palyusik *et al.* (1980) have described the results of feeding two lactating sows a diet containing pure zearalenone (40 mg/kg). In addition to various physiological effects attributable to the oestrogenic effect of this compound, analysis of the milk from these animals showed mainly β-zearalenol (> 80% of original toxin) and α-zearalenol (~15%) with only traces (0·5–1·3%) of unchanged zearalenone. The highest concentration of zearalenol found in milk was 0·79 p.p.m. The authors reported that the metabolites could be detected in the milk samples within 2 days of feeding the zearalenone and were still present 5 days after it had been removed from the diet.

Calculations quoted by Lovelace and Nyathi (1977) give possible daily intakes of zearalenone of 450 μg and 170 μg for rural farmers in Southern Province and

inhabitants of Lusaka, respectively. The figure for certain individuals is certainly much higher. Marasas *et al.* (1979), on the basis of animal data, considered that 500 μg/kg was a biologically significant dose of zearalenone. However, as has been indicated, there is a considerable variation in sensitivity between species and the toxicity of zearalenone in man is unknown, but based upon data from other primates (Hobson *et al.* 1977) is probably low. Ueno and Kubota (1976) suggested that zearalenone was mutagenic to a recombination-deficient line of *Bacillus subtilis*, but this could not be confirmed by Wehner *et al.* (1978), using *Salmonella typhimurium*.

Schoental (1979) has suggested that zearalenone and other *Fusarium* mycotoxins may have a role in the aetiology of tumours of the digestive tract and gonads in animals and man, and there has been speculation (see Martin and Keen 1978) that dietary oestrogens might be implicated in the high incidence of cervical cancer in certain areas of Southern Africa, e.g. Swaziland and Lesotho.

Mouldy corn from areas of the Transkei associated with high and low incidences of oesophageal cancer has been examined for mycotoxins (Marasas *et al.* 1979). Pooled samples from these areas showed no significant differences in the extent and nature of the *Fusarium* species present. However, when four sub-samples of hand-selected, visibly *Fusarium*-infected kernels were analysed, significant differences in the nature and extent of the infection were observed. All of the sub-samples contained zearalenone (ranging from 1500 to 10 000 μg/kg) but the mean level of the samples from the high-incidence area was 5750 μg/kg compared to 2750 μg/kg from the low-incidence area. Even larger differences were noted in the levels of deoxynivalenol, being 250 μg/kg and 2500 μg/kg in the low-incidence and high-incidence areas respectively. The authors concluded that before the potential threat to human health of these *Fusarium* metabolites in mouldy corn could be evaluated more detailed information was needed on their chronic effects and on whether any additive or synergistic effects might occur.

Other compounds claimed to possess oestrogenic activity

Examination of table 1 reveals additional compounds which have been claimed, with varying degrees of supportive evidence, to be responsible for the oestrogenic activity of the individual plant species shown. For example, Stob (1983) has suggested that the hormonal activity of carrots (Ferrando *et al.* 1961) may be related to the presence of 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (XXVIII, figure 10). However, this compound was isolated from cold-stored carrots and was apparently absent in the freshly harvested root (Sondheimer 1957). Little, if anything, is known about the effect of such storage on the uterotropic effect of this vegetable. Anethole (XXIX) was suggested by Zondek and Bergmann (1938) to be responsible for the oestrogenic activity of essential oils of fennel and anise, but more recent physiological studies on this compound (Sangster *et al.* 1984a, b) have not supported this. Three structurally related bitter acids, colupulon (XXX), lupulon (XXXI) and adlupulon (XXXII) have been identified in hops and proposed as the oestrogenic principles therein (Zenisek and Bednar 1960).

Feldman *et al.* (1982) described a protein in bakers' yeast (*Saccharomyces cerevisiae*) capable of binding 17β-oestradiol with high affinity; moreover, a chloroform extract of the same yeast cells was found to bind competitively to mammalian oestrogen receptor cells *in vitro*. Subsequently the same group (Feldman *et al.* 1984) showed this extract to possess uterotropic activity. If these findings are confirmed, high priority should be given to the isolation and identification of the active component(s), given the extensive use of this material in baking and fermentation. Only when its potency has

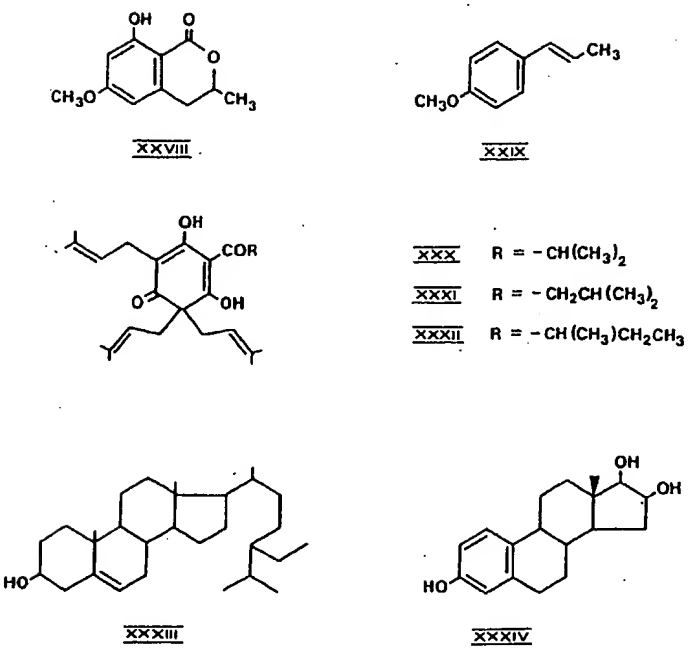


Figure 10.

been determined and its levels in food products have been established can the full significance of the above findings be ascertained.

According to Hassan *et al.* (1964), β-sitosterol (XXXIII) was one of the factors responsible for the hormonal activity of liquorice, although this has been questioned. Certainly if this and related compounds were to be confirmed as possessing such activity, then their ubiquity might well explain the hormonal properties of a range of common food plants, including onion and garlic and certain vegetable oils (Booth *et al.* 1960). Rather earlier, Costello and Lynn (1950) had tentatively identified the steroidal oestrogen, oestriol (XXXIV), as being present in liquorice. The role, and indeed the presence, of such steroidal hormones in the plant kingdom has been the subject of considerable controversy (Hewitt *et al.* 1980). As these workers have emphasized, early investigations into the hormonally active principles of plants were limited by relatively crude means of fractionation, isolation, characterization and bioassay. Consequently many of the initial claims for the occurrence of steroidal oestrogens in the plant kingdom were treated with scepticism. In 1966, Bennett *et al.* identified oestrone in date palm pollen by thin layer chromatography, and it was subsequently isolated from the same source (Amin *et al.* 1969). Pomegranate seed was claimed to be another source of this compound, with levels of 17 mg/kg being reported (Heftmann *et al.* 1966).

Whilst the presence of oestrone was confirmed by Dean *et al.* (1971), the measured levels were very much lower (4 μg/kg). 17β-Oestradiol could not be detected. In other cases (see Hewitt *et al.* 1980), workers were unable to isolate steroidal oestrogens from plant sources despite previous claims to the contrary. Such irreproducibility, limitations in analytical technique and methodology and the overriding concern that presence of such compounds, even when proved conclusively, might be a result of contamination meant that fundamental questions about the natural occurrence of such compounds in plants remained until recently (Van Rompuy and Zeevaar 1979), and the

failure of these workers to identify steroidal oestrogens in plant extracts using sophisticated modern techniques clearly identifies this area as a rewarding one for further interdisciplinary study. Work described in detail by Hewitt *et al.* (1980), using radioisotope incorporation studies and sensitive gas chromatography-mass spectrometric techniques, unequivocally revealed the presence of oestrone and oestradiol in French bean seedlings.

Residues of pesticides and insecticides may also be a source of uterotropically active compounds in the human diet. It has long been known that DDT and its analogues exhibit such activity (Fisher *et al.* 1952, Welch and Conney 1968) and recently Loeber and van Velsen (1984) have shown β-HCH, an isomer of lindane and a component of technical HCH, to have uterotropic activity. Although very weak (2×10^{-5} that of 17α-ethynyloestradiol), little is known about the effects of long-term exposure to trace amounts of such compounds.

Overview

Amongst the plants consumed by humans which have been reported to possess oestrogenic activity are onion, garlic, coffee, apple, parsley, sage, rhubarb, potato, radish, pea, cucumber, sugar beet, cabbage and mustard. These reports, originating from the early work of Dohrn *et al.* (1926) and Löve and Löve (1945), did not identify any of the active components, and were based upon methods of analysis which are now recognized to have limitations. Nevertheless it would seem desirable to re-examine some of these food plants using modern methods of analysis, in particular those like potato and cabbage, which are consumed regularly in relatively large amounts. The widespread use of vegetable oils also suggests that the claims that these are uterotropic be re-examined. Because of the evident variation in sensitivity to oestrogens exhibited by different species and strains of animal it would be desirable to standardize the uterotropic assay so that results from different laboratories and on different commodities/food plants could be more readily compared.

Inspection of table 6, taken from Verdeal and Ryan (1979), would seem to suggest that there is little risk associated with the intake of plant oestrogens. This is not necessarily the case, however, since little is known about the effects of long-term low-level exposure to these compounds (or their metabolites). Studies with human subjects would be desirable to determine whether or not normal levels of intake are associated with detectable physiological changes. This might provide objective predictions of the nature and extent of any changes which might occur in particular

Table 6. Human exposure to exogenous oestrogens.[a]

Source	Estimate of possible daily dose (μg diethylstilboestrol equivalents)
Morning-after pill	50 000
Birth control pill	2500
Post-hysterectomy replacement therapy	500–1000
Post-menopausal therapy	500
100 g beef liver (0·5 p.p.b. diethylstilboestrol)	0·05
100 g wheat (2 p.p.m. zearalenone)	0·2
20 g (d.w.) soyabean sprouts (70 p.p.m. coumestrol)	0·5
100 g French beans (2–10 p.p.b. oestradiol)	0·03–0·15

[a] Data from Verdeal and Ryan (1979) with permission.

'at-risk' sections of the population. Moreover, consideration should be taken of any medium or long-term changes in dietary habits which might be expected to increase the intake of such phytoestrogens; the increasing use of vegetable proteins in general and soya protein in particular and the introduction of soya milk products for infant feeding are two such examples (Setchell *et al.* 1984).

The importance of metabolic studies in determining the likely oestrogenic effect associated with the ingestion of isoflavones and resorcylic acid lactones is obvious, the effect depending as it does on the extent of that metabolism and the individual potencies of the metabolic products. The metabolism of the coumestans should be examined and the activities of the major isolated metabolites determined. Further studies should also be conducted on equol; for example, examining its effect *in vivo* and *in vitro*. Such studies will obviously depend upon the availability of methods of analysis for both the parent compounds and the metabolites. The examination of the possible carry-over of resorcylic acid lactones, and metabolites, following the feeding of *Fusarium*-infected diets or the implantation of zearalanol as growth stimulant would be desirable using such methods. The possible synergistic effect of different plant oestrogens or of plant and synthetic oestrogens should not be discounted (Kotsonis *et al.* 1975).

Attention should be given to programmes designed to limit or reduce the intake of plant oestrogens, whether by judicious selection of plant varieties or the optimization and improvement of agronomic, storage and processing conditions. Notwithstanding the inherent difficulties, a detailed study of the dietary factors associated with the high incidence of certain cancers in Southern Africa might provide useful information as to the role of dietary oestrogens. Indeed, a fuller understanding of the biological basis of the hormonal activity of the plant oestrogens considered above, particularly in farm animals and primates, would seem long overdue.

Future progress in this interesting and challenging area will largely depend upon the integrated efforts of workers from a variety of disciplines, including chemists, biochemists, toxicologists, pathologists, food technologists and plant breeders. As such it would seem to be particularly suitable for support from national and international food and health agencies.

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